THE REGULATION OF INTERLEUKIN-8 FROM MACROPHAGES BY ACUTE HYPOXIA AND HYPEROXIA: A ROLE IN THE PATHOGENESIS OF THE ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

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DECLARATION

This thesis was composed entirely by myself on the basis of work carried out between October 1996 and October 1999. Unless otherwise stated, all experiments were performed by myself under the supervision of Dr Seamas Donnelly and Professor Chris Haslett in the Rayne Laboratory, Respiratory Medicine Unit, University of Edinburgh.

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ABSTRACT

The acute respiratory distress syndrome (ARDS) is a catastrophic form of acute lung inflammation. Patients with ARDS require support on the intensive care unit (ICU) and the associated mortality approaches 50%. ARDS represents the severe end of a spectrum of lung injury that evolves over a period of hours or days in a subgroup of patients following a major insult such as multiple trauma, sepsis or aspiration. Professor Haslett's group in Edinburgh have undertaken clinical studies in patients in the very early at-risk period of ARDS, soon after the initiating insult. We have shown that in patients with multiple trauma, raised levels of intrapulmonary interleukin-8 (IL-8), but not other inflammatory cytokines, are associated with subsequent progression to ARDS ($n=56$, $P<0.001$). IL-8 is a potent chemoattractant and activator of neutrophils, considered to be the primary injurious cell in ARDS. The high IL-8 levels were detected within a few hours (range 0.75 - 4 hr) of the trauma incident. Immunohistochemical analysis implicated the alveolar macrophage as a potent source of intrapulmonary IL-8. The mechanisms by which IL-8 may be rapidly generated in this clinical setting are unknown.

Our clinical observations suggest that events occurring in the immediate aftermath of a trauma incident contribute to the generation of IL-8 in macrophages. I hypothesised that clinically relevant physiological events may include:

1) A neuro-endocrine 'stress' response to major trauma. This would result in the rapid intrapulmonary and systemic release of clinically relevant stress mediators including catecholamines and neuropeptides that may stimulate the macrophage to generate IL-8.
2) Acute tissue hypoxia and hyperoxia. By the time of sampling, the trauma victims were likely to have undergone a period of sustained tissue hypoxia secondary to head-injury, atelectasis and lung contusion and subsequent resuscitation with delivery of high flow oxygen. I hypothesised that hypoxia / hyperoxia was as direct multiple-stimuli or 'hits' to generate IL-8 in macrophages.

I aimed to test these hypotheses in studies of cultured human monocyte-derived macrophages and in a novel animal model of acute lung injury.

In human-monocyte derived macrophages, I have shown that the stress mediators adrenalin, substance P and macrophage migration inhibitory factor (MIF) do not increase IL-8 production at an early time-point (2 hr). Compared to normoxic controls, acute hypoxia ($PO_2 \sim 3.5$ KPa) increased IL-8 protein release by 1.8-fold by 2 hours and steady-state IL-8 mRNA expression by 30 mins. The multiple hit of hypoxia / hyperoxia was found to be a more potent stimulus for IL-8 generation than hypoxia or hyperoxia alone.

The effects of hypoxia / hyperoxia on IL-8 generation were studied in a rabbit model of acute lung injury. Localised bronchoscopic instillation of HCl into the left lower lobe of an anaesthetised ventilated rabbit resulted in significantly increased IL-8 mRNA and protein expression, neutrophil infiltration into alveolar airspaces and lung in the directly injured lung but not the contralateral 'indirectly' injured lung. Systemic hypoxaemia was induced by reduction in the inspiratory oxygen fraction. Compared to normoxic controls

(arterial PaO_2 ~ 11 KPa), acute hypoxia (PaO_2 ~ 5 KPa) for up to 2 hours increased intrapulmonary IL-8 mRNA but not protein expression in the acid-injured lung. Delivery of 100% oxygen for 2 hours (PaO_2 ~ 60 KPa) following acute hypoxia (a multiple-hit), increased both intrapulmonary IL-8 mRNA and IL-8 protein levels. The increase in IL-8 protein was attenuated if the reoxygenation phase was controlled to return arterial PO_2 to normoxic levels (~ 11 KPa).

The mechanisms by which hypoxia may rapidly increase IL-8 mRNA expression in monocyte-derived macrophages was further studied *in vitro*. The rapidity of the response (30 mins) suggested an increase in gene transcription. Electromobility gel-shift assay revealed that hypoxia increased nuclear levels of the IL-8 promoter-binding transcription factors AP-1 and CEBP-β, but not NF-κB, by 15 min exposure. Hypoxia induced macrophage expression of HIF-1α, a critical regulator of hypoxic adaptive responses. However cobalt chloride and desferrioxamine, HIF-1α-inducing hypoxia mimics, did not upregulate IL-8, suggesting that IL-8 transcription may be HIF-1 independent. Finally it was demonstrated that in contrast to IL-8, hypoxia inhibited expression of a panel of chemokines and cytokines including MCP-1, MIP-1α, MIP-1β and TNF-α. Both the pattern of chemokine expression and transcription factor activation with hypoxia differed from that induced by bacterial lipopolysaccharide (LPS), which potently activated NF-κB and upregulated several inflammatory genes.

These data support the hypothesis that acute hypoxia / hyperoxia act as multiple-hits in the generation of macrophage-derived IL-8 *in vitro* and intrapulmonary IL-8 *in vivo*, representing a potential mechanism for our observation of elevated alveolar IL-8 levels patients with multiple trauma that progress to ARDS. The observation that hypoxia alone rapidly and selectively increased IL-8 mRNA expression suggests that hypoxia may represent a 'priming' stimulus in macrophages, 'arming' the cell for a subsequent second hit such as hyperoxia. Furthermore, the specific chemokine response to hypoxia differs markedly with that observed with LPS implying potentially distinct adaptive responses to hypoxia and infection in the macrophage.

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This work is dedicated to my family.

Dear Sian, you may not want it, but this is for you.

Thanks Mohini and Nimmi for being there when I get home

and not caring in the slightest what I did at work.

I really needed that.

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ABBREVIATIONS

aa	Amino acids
ACCP	American College of Chest Physicians
Ad	Adrenalin
ALI	Acute lung injury
AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
bZIP	Basic region-leucine zipper
C/EBP	CCAAT/Enhancer-binding Protein
DARC	Duffy antigen receptor for chemokines
DFO	Desferrioxamine
DIC	Disseminated intravascular coagulation
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothrieitol
EB	Evans blue
EBr	Ethidium bromide
FiO_2	Fractional inspired oxygen
fMLP	Formyl-methionyl-leucyl-phenylalanine
HBS	HIF-1 binding site
HIF	Hypoxia inducible factor
HIP	Hypoxia inducible protein
HPA	Hypothalamic-pituitary-adrenal
ICU	Intensive care unit
IL	Interleukin
IP-10	Gamma-interferon inducible protein-10
IPTG	β-D-isopropyl-thiogalactopyranoside
LB	Liquid broth
LLL	Left lower lobe
LPS	Lipopolysaccharide

LTB_4	Leukotriene B_4
MCP-1	Monocyte chemoattractant protein-1
MDM	Monocyte-derived macrophage
MIP-1	Macrophage inflammatory protein-1
MIF	Macrophage migration inhibitory factor
MOF	Multi-organ failure
MODS	Multi-organ dysfunction syndrome
NF-κB	Nuclear factor -κB
PAF	Platelet-activating factor
PEEP	Positive end expiratory pressure
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
RLL	Right lower lobe
ROS	Reactive oxygen species
RT	Room Temperature
SCCM	Society of Critical Care Medicine
SIRS	Systemic inflammatory response syndrome
SNS	Sympathetic nervous system
SP	Substance P
TE	Tris-EDTA buffer
UTR	Untranslated region
V/Q	Ventilation / Perfusion

CHAPTER 1

INTRODUCTION

1.1. THE ACUTE INFLAMMATORY RESPONSE

The classical localised response to injury or infection has been recognised for at least 2000 years. Redness and swelling with heat and pain (*rubor et tumor cum calore et dolor*) were described in the writings of Celsus (30 B.C. to 38 A.D.) and together with loss of function (*functio laesa*), comprise the cardinal features of acute inflammation. Isolating the injured site, destroying the injurious agent and initiating healing are processes which, in evolutionary terms, must clearly be aimed at enhancing the hosts prospects of survival. Unless tightly regulated however, these processes are themselves liable to cause host injury. Indeed, it is this potential conflict that is believed to be central to the pathogenesis of inflammatory disease (Gallin et al., 1992)

Whilst the cardinal features of localised inflammation have long been recognised, the concept of a systemic inflammatory response to a significant injury or insult is comparatively new. In his writings in the 1850's, Claude Bernard had recognised that in order to sustain life, the constancy of the '*milieu intérieur*' must be maintained (Selye, 1956). This fundamentally important concept was termed homeostasis by Walter Cannon in the 1930's. Throughout the first half of the 20th Century, numerous terms were coined to embody a stereotypical response to a major insult, manifested by a disturbance of homeostatsis: '*Maladie postopératoire*', 'hemoclastic crisis', '*syndrome chimque d'histolse massive*', and '*le syndrôme malin de maladies infectueuses*' were all characterised by changes in blood pressure, heart rate, respiratory function, thermoregulation, and immune and metabolic activity in response to surgery, blood loss, trauma, burns and infection (Selye, 1956).

In the last 50 years, our understanding of this acute systemic response has advanced on two fronts. Firstly, there are now widely accepted definitions and classifications for the systemic inflammatory response and its complications, greatly aiding clinical studies. Secondly, the nature and function of many neuro-endocrine and immune mediators, and their cellular sources, is becoming clearer.

1.1.1 The syndromes associated with systemic inflammation

The term 'systemic inflammatory response syndrome' (SIRS) was introduced following a consensus conference of the American College of Chest Physicians and Society of Critical Care Medicine (ACCP/SCCM) a decade ago (Bone et al., 1992). It is defined as the response to a severe clinical insult and manifested by the presence of two or more conditions from a list of criteria relating to temperature, heart rate, respiratory rate and white blood cell (WBC) count (**Appendix 1A**). The term 'sepsis' is used to describe an identical syndrome, but in which infection can be identified as a cause.

Figure 1.1.1

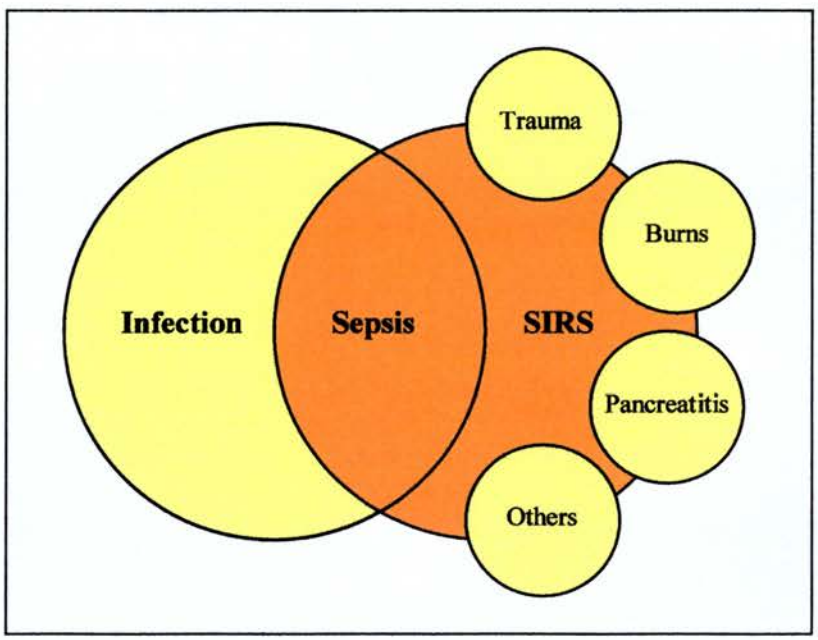


Figure 1.1.1 Relationship between major clinical insults and SIRS. Sepsis is infection complicated by SIRS. Adapted from Bone et al., 1995

Although the criteria used to define SIRS were based on consensus and not on cited clinical or experimental data, subsequent prospective studies have demonstrated that the early presence of SIRS and the presence of >2 criteria does confer an increase in patient mortality on the intensive care unit (ICU) (Rangel-Frausto et al., 1995; Malone et al., 2001). Virtually all patients that die in an ICU setting exhibit dysfunction or failure of one or more major organs. Indeed there is a virtually linear relationship between mortality

and number of organs failed (Moreno et al., 1999). Hence, SIRS may be viewed as a phase within a continuum, with the potential to progress from a heightened inflammatory state to an overwhelming inflammatory cascade, precipitating dysfunction in major organs such as the lung, kidney and liver. The ACCP/SCCM consensus committee of 1991 termed this state of altered organ function (or failure of homeostasis) the multi-organ dysfunction syndrome (MODS). No attempt was made to define or categorise 'organ dysfunction' specifically, though several scoring systems have subsequently been suggested (Nathens and Marshall, 1996). In the context of a severe systemic inflammatory response, the lung appears to be particularly susceptible to injury (Vincent et al., 1998). In its most catastrophic form, this is termed the acute respiratory distress syndrome (ARDS).

1.1.2 The acute respiratory distress syndrome

In 1967, Ashbaugh and colleagues described twelve patients on the intensive care unit with respiratory failure in whom i) the chest radiograph revealed bilateral diffuse alveolar shadowing, ii) hypoxaemia was refractory to oxygen therapy, iii) lung compliance was markedly reduced and iv) pulmonary oedema and hyaline membrane formation was prominent in the necropsy specimens of the seven that died (Ashbaugh et al., 1967). Soon after, this constellation of abnormal findings was termed the adult respiratory distress syndrome, to distinguish it from a similar pathological condition in neonates (Petty and Ashbaugh, 1971).

1.1.2.1. Definition of ARDS

The diagnostic criteria for diagnosing ARDS have evolved since Ashbaugh's original description. The central clinical features of respiratory failure with refractory hypoxaemia, pulmonary infiltrates secondary to leakage of proteinaceous fluid and 'stiff' lungs still apply, but have been both refined and clearly defined - a process critical for the purposes of epidemiological and therapeutic studies. It is recognised that ARDS represents the severe end of a spectrum of acute lung injury (ALI) and the attempts at diagnostic criteria reflect this. In 1988 Murray and colleagues proposed an expanded

definition of ARDS that allocated a lung injury score to individual cases based upon; chest radiograph, degree of hypoxaemia, PEEP (peak end-expiratory pressure – an indirect measure of lung compliance) and compliance itself if available. A score of 0 indicated no lung injury, 0.1-2.25 indicated mild to moderate injury and >2.5 indicated severe lung injury (ARDS). This scoring system has been widely used in studies of ALI / ARDS.

More recently the key features of this scoring system have been incorporated into a potentially simpler definition; The North American / European Concensus Criteria (Bernard et al., 1994)(**Appendix 1B**). This acknowledged that lung injury was a continuum from mild to severe and distinguished ARDS from acute lung injury on the basis of severity of impaired oxygenation alone. Furthermore, it emphasised that lung injury was associated with a precipitating or predisposing event.

1.1.2.2 Pathological features of ARDS

The various predisposing events associated with ARDS may be broadly separated into two main categories; in one, the lungs are injured **directly** by a disease process and in the other, lung injury occurs **indirectly** in the setting of a distant or systemic process (**Table 1.1.**).

Table 1.1.

Direct	Indirect
Aspiration of gastric contents	Sepsis
Pneumonia	Multiple Trauma
Smoke Inhalation	Acute Pancreatitis
Near Drowning	Burns
Pulmonary Emboli	Massive Transfusion
Pulmonary contusion	Reperfusion Injury

Table 1.1. Major insults or events predisposing to the development of ALI / ARDS

In the early stages of ARDS, there may be histological features that reflect the underlying precipitating event. For example, microthrombi may be observed where ARDS is associated with disseminated intravascular coagulation (DIC), or there may be histological evidence of aspiration or atypical pneumonia (Hasleton, 1996). Most of the early ultrastructural pulmonary changes are however similar regardless of the cause.

Injury to the type 1 alveolar epithelial cell, characterised by cytoplasmic swelling, membrane fragmentation and exposure of the underlying basement membrane, is considered an important, relatively early event in ARDS (Baclofen and Weible, 1977). The alveolar wall capillary endothelial cells also show evidence of swelling, vacuolation and focal necrosis (Baclofen and Weible, 1982). The relative importance of endothelial versus epithelial injury in the pathogenesis of ARDS is unresolved (Schnells et al., 1980). This may reflect the variety in the initiating insults that give rise to lung injury.

Widespread and prominent oedema of the alveolar walls and airspaces (lung leak) is a feature of established ARDS. This fibrin-rich oedema fluid forms 'hyaline membranes', possibly at sites of type 1 epithelial cell injury (Sevitt, 1974). Neutrophil infiltration is a prominent feature of established disease and there is compelling evidence that the neutrophil, through release of multiple histotoxic agents, is injurious to the lung parenchyma (Downey et al., 1999; Haslett et al., 2000). Both experimental and clinical studies have, however, demonstrated that neutrophil recruitment alone may occur without alteration in lung endothelial or epithelial permeability to protein (Wiener-Kronish et al., 1991; Martin et al., 1989).

1.1.2.3 The natural history of ARDS

The mechanisms by which an initially severe but potentially limited insult leads to catastrophic lung injury are not clearly defined. There are however characteristic features in the natural history that may provide insight into the pathogenesis of ARDS. Firstly, lung injury evolves over time and is not clinically apparent until several hours or days following the initiating insult. This 'latent period', during which most patients will exhibit SIRS, is particularly evident when ARDS follows an indirect insult such as

multiple trauma or sepsis. Secondly, not all patients with a predisposing disease will develop severe lung injury. The percentage of those at risk that progress to ARDS depends upon the cause; approximately 40-60% of patients with sepsis, 20-35% of trauma patients and 10-20% of patients with gastric aspiration (Fowler et al., 1983) progress to ARDS.

Finally, established ARDS is associated with a high mortality and to date no pharmacological therapy has convincingly been shown to reduce mortality (reviewed by Ware and Matthay, 2000). In the early 1980's figures of 65% or higher were frequently quoted (Milberg et al., 1995), and whilst this figure has fallen a little over the last 20 years, it remains over 50% in most centres. The majority of deaths are a consequence of MODS and sepsis, with less than a fifth attributable directly to respiratory failure (Montgomery et al., 1985). These characteristics of the disease have led some investigators to focus on the at-risk period of ARDS, offering the potential for identifying key mediators that may play a role in the development of severe lung injury. The at-risk period begins immediately after the onset of the initiating insult and as such the acute systemic response may be highly relevant to subsequent disease progression.

1.1.3. The systemic response to a major insult

A severe insult such as major trauma, infection or other conditions predisposing to ARDS, leads to a systemic co-ordinated host response. The principle aim is preservation of critical organ function achieved through maintenance of blood pressure, perfusion and energy supply. Known as the 'stress response' (Selye, 1936) or the 'fight or flight' reaction (a term used by physiologists such as Walter Canon in the 1930's), the physiological effects may be very rapid if in response to an acute, perceived injurious insult. This early response is orchestrated through the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis.

1.1.3.1. Interaction between the SNS and HPA following a major insult

The complex interaction or cross-talk between the HPA and the SNS is mediated via a range of neuro-endocrine and inflammatory cell mediators, and occurs at multiple levels both centrally and in peripheral tissues (**Figure.1.1.2**). For example, the SNS originates in the brainstem and included in its extensive network of innervation is lymphoid tissue and the adrenal glands. Activation of the SNS results in increased catecholamine expression, both localised release of noradrenalin from the sympathetic nerve fibres and systemic release of adrenalin from the adrenal medulla. Catecholamines are the principle end-products of the SNS and mediate tachycardia, vasoconstriction and critical rapid metabolic adaptations to severe injury. In addition, catecholamines have been shown to have a role in immunoregulation. In general, both adrenalin and noradrenalin are considered anti-inflammatory in nature, downregulating a range of proinflammatory cytokines and stimulating anti-inflammatory cytokines from antigen presenting cells and lymphocytes (reviewed in Elenkov et al., 2000).

A corresponding 'central' mediator in the HPA axis is corticotropin-releasing hormone (CRH), principally secreted in the paraventricular nucleus (PVN) of the hypothalamus. Corticotrophic cells in the anterior pituitary respond to CRH stimulation by releasing adrenocorticotropic hormone (ACTH) into the circulation which, in turn, stimulates cortisol synthesis in the adrenal cortex. Cortisol, the principle end-products of the HPA, is a critical mediator of the stress response. As with the catecholamines, cortisol predominantly exhibits anti-inflammatory properties.

There is a direct two-way communication between the HPA axis and the SNS at a central level, with CRH-secreting neurons projecting from the PVN into the brainstem-origin of the SNS and noradrenergic neurons projecting from the brainstem into the PVN. Hence, activation of one system tends to activate the other.

Figure 1.1.2

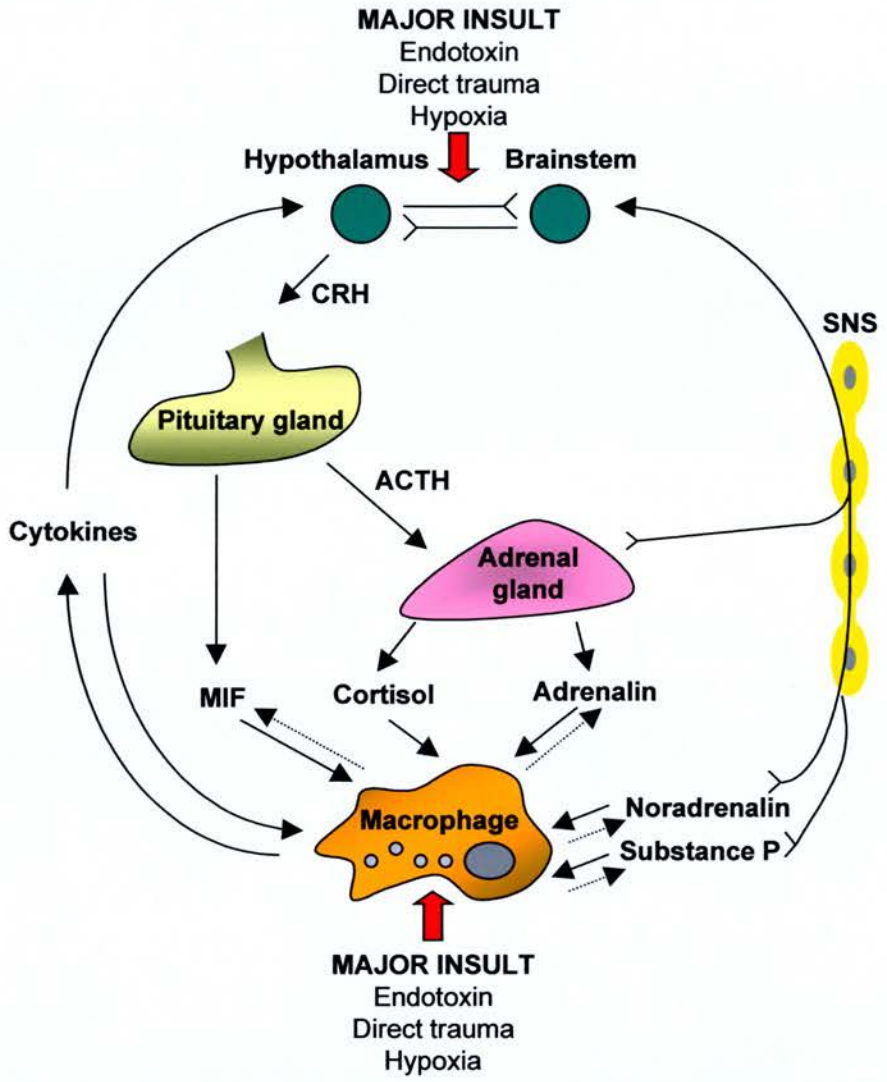


Figure 1.1.2 The interaction between the Hypothalamic Pituitary Adrenal axis (HPA) and the sympathetic nervous system (SNS) following a major insult.

Endotoxin, trauma and hypoxia have direct effects both on the brainstem and hypothalamus, and on peripheral tissue. In this figure, the cellular component in the tissues is represented by the macrophage. Tissue generated cytokines TNF-α, IL-1 and IL-6 stimulate the hypothalamus, which has bi-directional innervation with the brainstem origin of the SNS, resulting in dual activation. The neuro-endocrine mediators released following activation of the HPA / SNS, in addition to having widespread physiological effects (see text), also influence macrophage function. Finally, there is evidence that the macrophage is able to release a number of neuro-endocrine mediators (broken arrows), indicating potential autocrine activity.

1.1.3.2 Substance P

Although catecholamines and cortisol represent the major peripheral effectors of the stress response, there are a host of other candidates that may also play a regulatory role. Substance P (SP) is a neuropeptide stored in peripheral sensory nerve endings throughout the body. In sympathetic ganglia, SP is found co-localised with noradrenalin (Kessler, 1985) and may be essential to the maintenance of adrenal catecholamine secretion in response to stress (Livett et al., 1990). In contrast to the immunoregulatory activities of catecholamines and cortisol, SP is regarded as possessing proinflammatory properties (Payan, 1989) and these may be particularly relevant in the lung (Bozic et al., 1996; Kaltreider et al., 1997).

1.1.3.3 Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) was first described 35 years ago as a substance produced by activated T cells which inhibited the random migration of macrophages (Bloom and Bennett, 1966; David, 1966). It has subsequently been 'rediscovered' and amongst its varied activities, it is now viewed as a critical regulator in the stress response (Bucala, 1996; Donnelly et al., 2000). Pre-formed MIF granules have been identified co-localised to the same cells that release ACTH in the rat anterior pituitary and stimulation with CRF results in a dose-dependent release of MIF (Nishino et al., 1995). MIF is also released from activated inflammatory cells, including the macrophage (Calandra et al., 1994) and eosinophils (Rossi et al., 1998).

The potential importance of MIF in the stress response is demonstrated by the finding that it is able to directly counter-regulate the anti-inflammatory effects of glucocorticoids both *in vitro* and in a mouse model of endotoxaemia (Calandra et al., 1995). In clinical studies, raised circulating levels of MIF have been found in patients with sepsis (Lehmann et al., 2001) and trauma (Joshi et al., 2000). With regard to lung inflammation, intrapulmonary MIF levels are raised in patients with ARDS (Donnelly et al., 1996) and asthma (Rossi et al., 1998).

1.1.4. The classical early inflammatory mediators

At a tissue level, it is believed that a major insult such as infection, direct trauma or ischaemia causes localised cytokine release, which may then be amplified in a 'cytokine cascade' to generate high circulating levels of inflammatory mediators. Bacterial endotoxin or lipopolysaccharide (LPS) is a major component of the Gram-negative bacterial cell wall and is widely considered to be a principal mediator in the pathogenesis of SIRS and sepsis following in gram-negative infection (Morrison and Ryan, 1987; Gutierrez-Ramos and Bluethmann, 1997; Horn et al., 2000). Tumour necrosis factor (TNF) -α, interleukin (IL)-1 and IL-6 are the archetypal early inflammatory cytokines. The pleiotropic autocrine and paracrine activity of TNF-α and IL-1 place these cytokines at the summit of the perceived cytokine cascade (Tracey et al., 1986; Tracey and Cerami, 1994; Dinarello, 1992). Both cytokines are able to stimulate production of each other, IL-6 and multiple other inflammatory mediators, thus amplifying the inflammatory response. Independently, TNF-α, IL-1 and IL-6 are able to directly stimulate the hypothalamus and activate the HPA axis (**Figure 1.1.2**) (Chrousos, 1995; Imura, et al., 1991; Sapolsky et al., 1987).

Endotoxin is also able to activate the HPA axis, though this effect may be mediated through cytokines, particularly IL-6 (Perlstein RS et al., 1993). Finally, although TNF-α, IL-1β and IL-6 are considered the classical early inflammatory cytokines for the reasons described, this pattern of cytokine generation may not be relevant to all tissues. Within the lung, there is evidence that the chemokine interleukin-8 (IL-8) may be one of the earliest generated proinflammatory mediators. This is discussed in **Chapter 1.2.**

1.1.4.1 Tumour necrosis factor-α

TNF-α is a pleiotropic trimeric peptide and binds to 2 receptors; a 55 kD protein (TNFRI or CD120a) and a 75 kD protein (TNFRII or CD120b). Signalling occurs through these membrane bound receptors, but TNFRI and TNFRII are also expressed in an extracellular ('soluble') form, capable of binding TNF-α and limiting bioavailability. Human

monocytes and macrophages express both receptor types in soluble and membrane bound form, though the relative expression is stimulus dependent. In resting monocytes for example, membrane bound TNFRII is predominantly expressed and stimulation with LPS results in downregulated expression both in vitro and in circulating cells (Hart et al., 1996; Van der Poll et al., 1997).

In patients with pancreatitis and sepsis, serum TNF-α levels are characteristically raised, often within 30 mins of onset and peaking by 2 hours (DeGroot et al., 1989; Exley et al., 1992; Parsons and Moss, 1996). Multiple trauma appears to have a less consistent effect on circulating TNF-α (Ayala et al., 1990; Hoch et al., 1993). The 'toxic' effects of TNF-α include hypotension, fever, metabolic acidosis, coagulopathy and capillary leak (Natanson et al., 1989; Van der Poll et al., 1991), all phenomena associated with SIRS and the genesis of MODS.

1.1.4.2 Interleukin-1

There are two well characterised IL-1 proteins, termed IL-1α and IL-1β. Although products of two distinct genes, both cytokines possess a similar wide spectrum of activity. IL-1 binds several cell surface receptors, the two most prominent being IL-1RtI (p80, CDw 121a) and IL-1RtII (p68, CDw 121b) (Dinarello, 1992). Monocytes express both receptors, though signalling only occurs through IL-1RtI (Uhl et al., 1989; Granowitz et al., 1992). IL-1RtII has a regulatory function, inhibiting cell responses to IL-1 (Neumann et al., 2000). IL-1 converting enzyme (ICE), or caspase-1, is an enzyme required for the post-translational modification of inactive IL-1β (30 KDa) to active IL-1β (17 KDa).

The proinflammatory properties of IL-1 are akin to those of TNF-α and elevated systemic IL-1 is frequently detected in sepsis, though classically highest levels are reached after the TNF-α peak. In other conditions associated with SIRS such as trauma and / or haemorrhage however, systemic IL-1 is frequently undetectable for several hours or days following the insult (Rodrick et al., 1986; Hoch et al., 1993).

1.1.4.3 Interleukin-6

A characteristic feature of the response to a major inflammatory stimulus is the systemic elevation of a large number of serum proteins, broadly termed the acute phase proteins (Gabay and Kushner; 1999). The precise function of many of these upregulated proteins remains uncertain, though some, for example C-reactive protein, are known to play important regulatory roles in the inflammatory process (Xia et al., 1997; Heuertz et al., 1997). IL-6 is considered the chief stimulator of acute phase protein production (Gauldie et al., 1987), in addition to its functions as a pyrogen and inducer of IL-1 and TNF-α.

Peak serum levels of IL-6 following an inflammatory insult occur several hours after peak TNF-α and IL-1 expression. High levels have been consistently detected following sepsis, pancreatitis and trauma and of the three classical cytokines, circulating IL-6 correlates most closely with severity of illness and outcome in both trauma and sepsis (Gabay and Kushner, 1999).

1.1.4.4 Endotoxin

Endotoxin or LPS consists of a structurally conserved core lipid component, glycolipid A, and an outer component of O- and R-polysaccharide antigens (Wardle, 1997). It is the glycolipid A portion which forms the toxic moiety of the LPS macromolecule. Intravenous injection of low-dose endotoxin in humans results in most of the haemodynamic, metabolic and immune responses associated with SIRS (Martich et al., 1991; Dinarello 1991; van der Poll and Deventer, 2000). In patients in whom there is a clearly identifiable source, such as perforated bowel or renal or pulmonary infection, endotoxin is generally accepted as playing a central role in the development of SIRS, septic shock and potentially MODS (Morrison and Ryan, 1987; Bone, 1996).

Depending upon the nature of the insult and the injured organ, a variety of cells may respond to cytokine or endotoxin stimulation and contribute to the generation

of inflammatory mediators. However, the macrophage may have a central role, and this is discussed in **Chapter 1.3.**

1.1.5. Mediators in patients at-risk of ARDS

Based on current understanding of the development of SIRS and progression to lung injury, a number of investigators have attempted to identify mediators of potential pathological or prognostic significance both in the blood or alveolar airspaces of patients at-risk of ARDS (Pittet et al., 1997; Hirani and Donnelly, 1997). Interpretation of data from such studies requires an awareness of their potential limitations, in particular limitations which relate to practical obstacles that hinder studies of critically-ill patients at-risk of ARDS.

Ideally, at-risk patients should be identified as early as possible following the initiating insult. This would provide the widest 'window of opportunity' in which to measure potentially important mediators before the onset of ARDS. Furthermore, since ARDS is regarded as the severe end of a continuum of lung injury, targeting patients early would tend to recruit a more homogenous population with relatively mild injury. Finally, the perception of an inflammatory cascade suggests that the pathways involved become progressively more complex and divergent 'downstream' of the initiating insult. The search for key regulatory mediators may be more rewarding early in the disease process.

In a clinical setting however, targeting patients early is fraught with logistical difficulties. Defining the 'at-risk' period is relatively simple if the initiating insult is major trauma or surgery, but not in conditions in which the time of onset is likely to be imprecise as in sepsis or pancreatitis. Recruiting acutely critically-ill patients, in whom the priority is resuscitation and supportive care, may raise important issues of consent. In a number of studies, patients have been recruited after admission to the intensive care unit and requiring ventilatory support. Amongst these patients, there already exists a spectrum of lung injury, though not yet severe enough to satisfy diagnostic criteria for ARDS. A further dilemma, and one which may explain many of the apparent discrepancies in

results between studies, is that the risk factors for ARDS are heterogenous and the pathogenesis of ARDS may reflect this.

Patients with multiple trauma may represent a group particularly salient to studies in the pathogenesis of ARDS. The onset of the initiating insult is sudden and easily defined. Patients are frequently immediately intubated in order to maintain a patent airway. This allows relatively easy access for bronchoalveolar lavage (BAL) sampling very soon after the trauma episode. **Table 1.1.2** summarises studies of biological mediators in patients at-risk of ARDS following major trauma. Included is data from our own studies of ARDS, in which a particular focus was placed in targeting patients early in the disease process. In an initial study of 29 patients at-risk of ARDS, BAL levels of the chemokine interleukin-8 (IL-8) were significantly higher in the 7 patients who progressed to ARDS compared to the 22 that did not (Donnelly et al., 1993). There was no significant association with plasma IL-8 levels. Immunostaining studies of BAL cells revealed that the alveolar macrophage was a significant source of the rapidly elevated IL-8 detected. The at-risk population in this study however, represented a heterogeneous group of patients with major trauma, pancreatitis or perforated bowel.

With this in mind, we studied 56 consecutive patients (median age 48 years, range 18–89) presenting to the Edinburgh Royal Infirmary with major trauma and requiring intubation in the Accident and Emergency Department (Hirani et al., 2001). The median injury severity score (ISS, Baker and O'Neill, 1976) was 25 (range 16-66). A chest radiograph was taken and reviewed within 4 hours of admission for all patients. No patients had radiographic evidence of ARDS at this time. BAL sampling was performed at a median time of 95 mins (range 30–240) after the trauma event. Subsequent development of ARDS was defined using the European / American Consensus criteria (Bernard et al., 1994). Endotoxin levels and a range of chemokines and cytokines were measured in BAL fluid and plasma. Total BAL protein levels and BAL differential cell counts were determined. Seventeen of the 56 trauma patients subsequently progressed to ARDS and the mean BAL IL-8 level was significantly higher in these patients compared to those who did not (1425 ± 1841 v 206 ± 288 pg/ml)($P=0.0001$).

Table 1.2

Biological Mediator		Measured in	Association with ARDS	References
Cytokines	TNF-α	Blood	-	Parsons et al., 1992; Rouman et al., 1993; Hirani et al., 2001
		BAL		
	IL-1β	BAL	-	Hirani et al., 2001
	IL-8	Blood	-	Jorens et al., 1992
		BAL	+	Jorens et al., 1992
			+	Hirani et al., 2001
	Anti-IL-8 antibody	BAL	+	Kurdowska et al., 2001
Bacterial products	Endotoxin	Blood	-	Parsons et al., 1989; Donnelly et al., 1994A
		BAL	-	Hirani et al., 2001
Complement	C5a	Blood	-	Duchateau et al., 1984
Markers of neutrophil / endothelial activation	Elastase	Blood	+	Donnelly et al., 1995;
	E / P- selectins	Blood	+	Donnelly et al., 1994;
	L-selectin	Blood	+	Stengel et al., 2001
	vWf-Ag	Blood	-	Moss et al., 1996
	CD11b / CD18	Blood	+	Simms et al., 1991
Protein leak	Albumin	Urine	+	Gosling et al., 1994; Pallister et al., 1997
Surfactant proteins	SP-A	Blood	+	Pison et al., 1992
Iron metabolism	Ferritin	Blood	+	Sharkey et al., 1999

Table 1.2 Biological mediators measured in patients with major trauma at-risk of ARDS. Studies included are those in which the entire study population comprised trauma patients, or in which analysis of a subgroup of trauma patients was performed. Although several studies have demonstrated association with the development of ARDS, there have been no large prospective studies.

In contrast there was no significant difference in BAL levels of TNF-α (P=0.37), IL-1β (P=0.13), MIP-1α (P=0.49), MIP-1β (P=0.7) and MCP-1 (P=0.08) between the two groups. BAL endotoxin levels were also not significantly different between groups (P=0.80) and in 7 of the 17 patients who progressed to ARDS, endotoxin levels were at the lower limit of detection (2.5 pg/ml). Plasma endotoxin levels in patients who did and did not progress to ARDS were also not significantly different (5.6±3.1 v 4.7±3.2, P=0.65). A correlation was observed between degree of patient hypoxia at admission to casualty, as manifested by reduced PaO_2/FiO_2, and raised BAL IL-8 levels (r= -0.56, P<0.001).

Thus, our original observation that IL-8 was associated with the development of ARDS was confirmed in a larger population of at-risk trauma patients. Importantly, this association was not observed with a range of other cytokines, chemokines or endotoxin. Interleukin-8 is a potent neutrophil chemokine, and its properties are discussed in **Chapter 1.2.**. A correlation was observed between degree of patient hypoxia at admission to casualty, as manifested by reduced PaO_2/FiO_2, and raised BAL IL-8 levels (r= -0.56, P<0.001).

1.2 THE CHEMOKINES

1.2.1 Classification of chemokines

The chemokines (chemotactic cytokines) are group of small proteins (8-12 kDa) that play a role in attracting leukocytes and regulating recruitment to tissues during inflammation (reviewed by Luster, 1998; Keane and Strieter, 2000). Over 40 members of the chemokine family have been identified to date, exhibiting 20-70% homology in amino acid sequence. They are classified based on the relative position of two highly conserved NH_2-terminal cysteine residues. In the CXC or α-family, the two cysteines are separated by one non-conserved amino acid residue and in the CC or β-family the two cysteines are in juxtapostion. These two families are by far the most extensively characterised. The only representatives outside of the α and β-families are lymphotactin (a C chemokine) and fraktalkine (a CXXXC chemokine). **Table 1.3** lists the majority of human chemokines identified.

Table 1.3

Human Chemokine Families	
CXC Chemokines	CC Chemokines
Interleukin-8 (IL-8)	Monocyte chemotactic protein (MCP)-1,2,3,4,5
Epithelial-derived neutrophil activating protein (ENA-78)	I-309
Growth-related oncogene (GRO)-α, β, γ	Macrophage inhibitory protein (MIP)-1α,1β,1δ,3α,3β
Granulocyte chemotactic protein (GCP)-2	Regulated on activation normal T cell expressed and secreted (RANTES)
Beta-thromboglobulin (β-TG)	Eotaxin
Neutrophil activating protein (NAP)-2	Macrophage-derived chemokine (MDC)
Platelet factor 4 (PF4)	Haemofiltrate CC chemokine (HCC)-1,2,4
Interferon-γ-inducible protein 10 (IP-10)	Thymus and activation-regulated chemokine (TARC)
Monokine induced by interferon-γ (MIG)	Thymus-expressed chemokine (TECK)
Stromal cell-derived factor-1 (SDF-1)	6-cysteine chemokine (6Ckine)
Interferon inducible T cell alpha chemoattractant (ITAC)	Dendritic cell chemokine-1 (DC-CK1)
B cell-attracting chemokine-1 (BCA-1)	Pulmonary and activation-regulated chemokine (PARC)
Lipopolysaccahride-induced CXC chemokine (LIX)	Secondary lymphoid-tissue chemokine (SLC)
C Chemokines	CXXXC Chemokine
Lymphotactin-α,β	Fraktalkine

Table 1.3. The classification of chemokines

The CXC chemokines are further subdivided on the basis of the presence or absence of a structure/function domain near the N-terminal: CXC chemokines harbouring the sequence glutamic acid-leucine-arginine ("ELR" motif) are chemoattractants for neutrophils and possess angiogenic activity whilst those that do not are mononuclear cell chemoattractants and inhibit angiogenesis (Strieter et al., 1995).

1.2.2. Chemokine Receptors

Chemokine activity is mediated through specific G-protein-coupled receptors (GPCR). The functional unit of this family of cell-surface receptors comprises a seven transmembrane receptor coupled to the heterotrimeric G proteins. Currently at least five human CXC chemokine receptors (CXCR1 through CXCR5), ten CC chemokine receptors (CCR1 through CCR10), a C chemokine receptor (CR1) and a CXXXC chemokine receptor (CXXXCR1) have been cloned and characterised (Murphy, 1994; Premack and Schall, 1996; Keane and Strieter, 2000). In recent years, the biology of chemokine receptors has attracted tremendous interest, fuelled by the seminal observation that CCR5 represented a cofactor mediating HIV-1 entry into CD4+ cells (Feng et al., 1996; Dragic et al., 1996; Deng et al., 1996).

The varied expression of chemokine receptors both between and within cell types adds a further level of complexity to the inflammatory pathway. For example, the CXCR1 receptor is restricted to neutrophils and appears to mediate IL-8 signalling alone. In contrast, CCR3 is expressed on eosinophils, basophils and dendritic cells and binds RANTES, eotaxin, MCP-3, MCP-4 and MIP-1α.

1.2.3. Interleukin-8 – An archetypal CXC chemokine

In 1987, Yoshimura and Matsushima isolated a 10 kD polypeptide from LPS-stimulated human monocyte supernatants. The protein exhibited potent neutrophil chemotactic activity, hence the original term monocyte-derived neutrophil chemotactic factor (MDNCF). The cDNA was subsequently cloned independently by several investigators (Schmid and Weismann, 1987; Matsushima et al., 1988) and the protein was

subsequently renamed IL-8 (Balkwill and Burke, 1989). Thereafter this chemokine has become the most extensively studied in human disease.

1.2.3.1. Structural characterisation of IL-8

IL-8 is generated in a precursor form, consisting of 99 amino acids with a signal peptide in the hydrophobic N-terminal region (Matsushima et al., 1988). Subsequent cleavage results in a 72-amino acid ($IL\text{-}8_{72\text{-}aa}$) or a 77-amino acid ($IL\text{-}8_{77\text{-}aa}$) non-glycosylated protein. The former is the predominant active form released from stimulated monocytes, macrophages and neutrophils. $IL\text{-}8_{77\text{-}aa}$ is the predominant form secreted from endothelial cells (Hebert et al., 1990). There is evidence that the functional characteristics between the two forms differ: In contrast to $IL\text{-}8_{72\text{-}aa}$ the 77-aa peptide exhibits comparatively weak neutrophil chemoactivity (Nourshargh et al., 1992), is pro-apoptotic in leukaemic cells (Terui et al., 1998) and may indeed have anti-inflammatory properties (Gimbrone et al., 1989). However, the *in-vivo* relevance of these differences is not clear. Thrombin and plasmin have been shown to cleave 77-aa IL-8 to the 72-aa form (Hebert et al., 1990; Nakagawa et al., 1991). The proteolytic processing of IL-8 may be relevant to the *in vivo* activity of IL-8.

The three dimensional structure of IL-8 represents a short N-terminus followed by three anti-parallel β sheets linked together with loops and by a β turn to the α helix which constitutes the carboxyl terminal (Clore et al., 1990). The sites susceptible to cleavage by neutrophil elastase, cathepsin G and proteinase 3 reside in the region of the β sheets and in the folded molecule are inaccessible to proteinases (Pardrines et al., 1994). In the micromolar range, IL-8 is a functionally active dimer. However it has been shown that at physiological concentrations (pico- and nanomolar range), IL-8 is found in a functionally active monomeric form (Burrows et al., 1994; Rajarathnam et al., 1994)

1.2.3.2. Cellular sources of IL-8

A wide variety of cells have the capacity to secrete IL-8 (Mukaida et al., 1998). Monocytes and macrophages are potent sources, particularly in response to

proinflammatory mediators such as TNF-α, IL-1 and LPS (Yoshimura et al., 1987; Metinko et al., 1992; Peveri et al., 1988). IL-8 responsive-cells, notably neutrophils, also generate IL-8, leading to potential autocrine activity (Bazzoni et al., 1991; Siddiqui et al., 1999).

1.2.3.3. IL-8 and cell surface receptor binding

IL-8 has been shown to bind to three distinct families of receptor: (i) The CXC receptors; (ii) the Duffy antigen receptor for chemokines (DARC); (iii) the heparan sulphate proteoglycans. The CXC receptors exhibit restricted expression and transduce intracellular signalling upon binding. In contrast, DARC and proteoglycan receptors are ubiquitously expressed and bind IL-8 without associated signalling.

(i) CXC Receptors

IL-8 binds to two members of the CXC family of receptors: CXCR1 (IL-8RA) is relatively selective for IL-8, known only to bind GCP-2 of the other chemokines; CXCR2 (IL-8RB) in addition to IL-8 also binds GCP-2, GRO-α,β and γ, NAP-2, ENA-78 and LIX (Luster, 1998). Whilst IL-8 receptors have been reported on a variety of inflammatory cells, it is overwhelmingly the neutrophil that exhibits activation in response to IL-8. Approximately 20,000 high affinity (K_d $8x10^{-8}$ M) IL-8 receptors are expressed per neutrophil (Oppenheim et al., 1991). Interaction with IL-8 occurs via two distinct domains, with subsequent binding and signalling proceeding in two distinct phases (Wu et al., 1996).

(ii) Duffy antigen receptor for chemokines (DARC)

The Duffy antigen is a transmembrane spanning protein present on red blood cells (RBCs) that exhibits high binding affinity for the malarial parasite *Plasmodium vivax* (Miller et al., 1976). The role of DARC expression on RBCs is uncertain, but one suggestion is that these cells serve as a 'sink', mopping up excess chemokines. In support of this, DARC knockout mice have been shown to exhibit an exaggerated inflammatory response to endotoxin (Dawson et al., 2000). In 1993, Horuk et al. demonstrated that this receptor also bound a range of CXC and CC chemokines including IL-8. Subsequently

DARC was also detected on postcapillary venule endothelial cells and found to bind to a number of chemokines (Hadley et al., 1994).

(iii) Heparan proteoglycans

Heparan and heparan sulphate are negatively charged proteoglycan constituents of cell surfaces and extracellular matrix. As with DARC, heparan sulphate proteogycans have been shown to bind several chemokines including MIP-1β (Tanaka et al., 1993), platelet factor (PF)-4 (Stringer and Gallagher, 1997) and IL-8 (Baggiolini et al., 1994; Ramdin et al., 1998; Dunzendorfer et al., 2001).

It has been postulated that endothelial-expressed DARC and heparan sulphate proteoglycans serve to capture chemokines in the extracellular matrix, raising local concentrations. In the case of IL-8, expression on endothelial surfaces may play a critical role in recruiting neutrophils to the inflammatory site. (Rot et al., 1996; Middleton et al., 1997).

1.2.3.4. The role of IL-8 in inflammatory cell recruitment

Neutrophil extravasation from the bloodstream, across intact endothelium and into the inflammatory site is a tightly regulated process, evolving through a series of coordinated events (Nourshargh and Williams, 1995). These include: 1) phenotypic changes in the neutrophil and / or endothelium resulting in altered expression of adhesion molecules, 2) tethering and rolling of the neutrophil along the endothelium, 3) firm adherence of the progressively flattened neutrophil to the cell surface and 4) regulated passage of the neutrophil through interendothelial cell junctions, across the perivascular basement membrane and into the interstitium (Butcher, 1991; Springer, 1994).

In vitro IL-8 induces directed neutrophil migration along a concentration gradient (chemotaxis) and upregulation of neutrophil integrins (Detmers et al., 1990; Divietro et al., 2001). Human studies have revealed that the entire process of neutrophil recruitment can be emulated by injection of IL-8 into tissue (Colditz et al., 1990; Swensson et al., 1991; Leonard et al., 1991). Animal studies of IL-8 are partially restricted in that rodents

do not appear to express this chemokine. Mice do, however, express an IL-8 receptor homologue, genetic deletion of which severely impairs neutrophil transmigration following intraperitoneal thioglycollate injection (Cacalano et al., 1994). In transgenic mice engineered to over-express IL-8, circulating levels of the chemokine are constitutively raised and neutrophils accumulate in the pulmonary, splenic and hepatic vascular beds. Neutrophil extravasation following an inflammatory stimulus such intraperitoneal thioglycollate instillation is impaired due, it is postulated, to the absence of a significant IL-8 gradient (Simonet et al., 1994).

In contrast to rodents, the pattern and array of chemokine expression in rabbits is thought to be similar to that of humans (Fox-Dewhurst et al., 1997; Wuyts et al., 2000). The rabbit expresses IL-8 (82% homology with human IL-8) and both IL-8 receptor homologues (Beaubien et al., 1990; Prado et al., 1994). Numerous rabbit models of disease have clearly implicated IL-8 in the pathogenesis of neutrophil-associated tissue injury (Wada et al., 1994; Folkesson et al., 1995; Harada et al., 1996; Matsumoto et al., 1997; Nakamura et al., 2000).

There is therefore considerable evidence to support central role for IL-8 in neutrophil recruitment. Chemotaxis is, however, conceptually difficult to conceive in an *in vivo* setting. Constant blood flow would prevent the establishment of a persistent soluble chemoattractant gradient at the blood-endothelial cell interface (Colditz et al., 1985; Tanaka et al., 1993). Rot and colleagues have performed a series of elegant studies in which radiolabelled IL-8 was injected intradermally in rabbits and into viable human skin *in vitro*, and its 'journey' monitored. It was observed that within 30 min the IL-8 had been transported from the extravascular tissue across the venular endothelium and was present on the luminal surface of endothelial cells (Middleton et al., 1997). This process involved internalisation of the IL-8 into the endothelial cell, incorporation into smooth membrane-bound plasmalemmal vesicles and immobilisation on the luminal surface. This latter event is thought to occur through the interaction of the non-signalling C-terminus of the IL-8 molecule with endothelial heparan sulphate or possibly DARC (Rot et al., 1996). These studies suggest a mechanism for coordinated IL-8 – neutrophil interactions at the endothelial surface. The validity of this concept in neutrophil recruitment to the lung is

unproven. There is evidence that some of the cellular and molecular mechanisms proposed for neutrophil migration in the systemic vasculature may not applicable to the pulmonary circulation (recently reviewed by Wagner and Roth, 2000)

Finally, whilst it is accepted that IL-8 is predominantly associated with neutrophil recruitment, recent studies have revealed a role for IL-8 in the adhesion of eosinophils (Ulfman et al., 2001) and monocytes (Gerszten et al., 1999) to endothelium. Hence, IL-8 may play be an important mediator in a wide range of inflammatory diseases.

1.2.3.5. Role of IL-8 in angiogenesis

Angiogenesis, the growth of new blood vessels from existing vessels and capillaries, is a process critical to normal physiological development, growth and repair. Dysregulated angiogenesis, however, can result in catastrophic host damage and is implicated in the pathogenesis of fibroproliferative disease, tumour growth and chronic inflammation (Folkmann and Brem, 1992; Folkmann, 1995; Jackson et al., 1997; Majno, 1998).

An array of important angiogenic regulators has been described (comprehensively reviewed by Griffioen and Molema, 2000). In the chemokine superfamily, CXC chemokines containing the ELR motif (IL-8, ENA-78, GRO-α,β.γ) in general exhibit angiogenic activity, whilst those without the ELR motif (PF4, IP-10, MIG) are angiostatic (Moore et al., 1998). IL-8 mediates endothelial cell chemotactic and proliferative activity (Strieter et al., 1992) and neovascularisation *in vivo*, independent of its neutrophil recruitment properties. Human non-small cell lung cancer (NSCLC) growth is significantly attenuated by a neutralising anti-IL-8 antibody treatment strategy in a murine model (Arenberg et al., 1996). It has been postulated however that it is the net balance between angiogenic versus angiostatic CXC chemokines that may determine disease activity and progression (Arenberg et al., 1996A; Keane et al., 1997; Moore et al., 1998).

1.2.3.6. IL-8 in human disease

Since its initial description, IL-8 has been implicated in the pathology of a number of diseases. The vast majority of these are primarily disorders of inflammation. (**Table 1.2.1**)

1.2.3.7. IL-8 in sepsis and MODS

Intravenous administration of LPS in primates or healthy human volunteers results in a rise in circulating IL-8 by 1 hour, peaking at 2 hours (Van Zee et al., 1991; Martich et al., 1991). Studies of patients with sepsis have consistently shown high serum IL-8 levels, that either independently or in combination with other markers of inflammation, have predictive value for progression to multi-organ failure or death (Marty et al., 1994; Fujishima et al., 1996; Takala et al., 1999; Lin et al., 2000; Slotman, 2000). In a rabbit model of septic shock, pre-treatment with an anti-IL-8 antibody improved survival (Carvalho et al., 1997; Yokoi et al., 1997). However there have been no published studies to date of anti-IL-8 therapy in sepsis or MODS in humans.

1.2.3.8 IL-8 in inflammatory lung diseases

It is in lung inflammation, and in particular pulmonary diseases in which the neutrophil is a prominent feature that IL-8 is most strongly associated (**Table 1.2.1**). Whilst it is possible that IL-8 represents a marker of intrapulmonary inflammation, the known biological properties of IL-8 and the data from animal studies suggest a causal association between IL-8, neutrophil recruitment and hence the potential for lung injury. Several lines of evidence from clinical studies further support this notion.

Our own clinical studies have identified an association between raised intrapulmonary IL-8 levels and the progression to ARDS (Donnelly et al., 1993; Hirani et al., 2001). Other investigators have also identified this chemokine as an early marker of inflammation, and have demonstrated raised intrapulmonary levels present before the onset of neutrophil influx in neonatal lung disease (McColm and McIntosh, 1994; Munshi et al., 1997;

Ghezzi et al., 1998) and in cystic fibrosis (Khan et al., 1995). In a recent study of lung transplant patients, our group reported that raised intrapulmonary IL-8 levels in donor lungs were significantly associated with subsequent development of graft dysfunction in the recipients (Fisher et al., 2001). Studies have revealed that persistently raised alveolar IL-8 levels are associated with non-resolution of ARDS and death (Miller et al., 1992; Baughman et al., 1996). Although the related CXC chemokines, particularly ENA-78 and the Gro-family, have neutrophil chemotactic properties, IL-8 is generally regarded as being the more potent and biologically relevant in acute lung injury. Neither ENA-78 nor Gro-α were found to be associated with the development of ARDS (Donnelly, unpublished data) or graft dysfunction following transplant (Fisher et al., 2001).

Goodman et al., (1996) have demonstrated that interleukin-8 may be responsible for over 50% of the neutrophil chemotactic activity of BAL fluid in patients with established ARDS, with comparatively little contribution from other CXC chemokines. Clearly therefore factors other than IL-8 also play a role in pulmonary neutrophil chemotaxis. Leukotiene B_4 (LTB_4), a myeloid-cell product derived from arachadomic acid, is chemotactic for neutrophils (Ford-Hutchinson et al., 1980) and macrophages (Hubbard et al., 1991). In chronic inflammatory lung disease, namely COPD and bronchiectasis, LTB_4, together with IL-8, is regarded as a major neutrophil chemoattractant (reviewed in Hill et al., 2000). The complement protein C5a, a product of classical complement cascade activation, is also potent neutrophil chemoattractant (Strunk et al., 1988). In patients at-risk of ARDS, plasma and BAL levels of LTB_4 have been found to be higher than in controls (Stephenson et al., 1988; Antonelli et al., 1994). Similarly, raised plasma levels of C5a have been reported in at-risk patients (Weinberg et al., 1984; Duchateau et al., 1984). Neither mediator has been shown to predict progression to ARDS in at-risk patients (**Table 1.1.2**).

Table 1.2.1

DISEASE	REFERENCE
ARDS	Jorens et al., 1992; Miller et al., 1992; Chollet-Martin et al., 1993, Donnelly et al., 1993; Torre et al., 1993; Hirani et al., 2001
Asthma	Marini et al., 1992; Folkard et al., 1997; Gibson et al., 1997
Chronic brochitis and emphysema	Richman-Eisenstat et al., 1993; Hill et al., 1999; Crooks et al., 2000
Bronchiectasis	Mikami et al., 1998
Idiopathic pulmonary fibrosis	Lynch et al., 1992; Southcott et al., 1995; Keane et al., 1997; Panteladis et al., 1997
Cystic fibrosis	Khan et al., 1995
Bacterial pneumonia	Rodriguez et al., 1992; Bohnet et al., 1997
Post-transplant lung injury	Fisher et al., 2001
Neonatal bronchopulmonary dysplasia	McColm and McIntosh, 1994; Groneck et al., 1994; Tullus et al., 1996; Munshi et al., 1997; Ghezzi et al., 1998
Acute chest syndrome in sickle cell disease	Abboud et al., 2000
High Altitude Pulmonary Oedema	Kubo et al., 1996
Lung cancer	Strieter et al., 1995; Arenberg et al., 1996A and 1996B; Yatsunami et al., 1997; Yuan et al., 2000
Sepsis	Hack et al., 1992, Marty et al., 1994; Endo et al., 1995; Fujishima et al., 1996; Takala et al., 1999; Lin et al., 2000
Multi-organ dysfunction syndrome	Hamano et al., 1998; Slotman et al., 2000; Holzheimer et al., 2000
Glomerulonephritis	Wada et al., 1994; Lai et al., 1996; Ralston et al., 1997; Sekikawa et al., 1998
Cerebral infarction or head injury	Kostulas et al., 1998; Whalen et al., 2000
Inflammatory bowel disease	Mahida et al., 1992; Gibson and Rosella, 1995
Pancreatitis	Gross et al., 1992; Chen et al., 1999
Rheumatoid arthritis	Brennan et al., 1990; Deleuran et al., 1994; Woods et al., 2000
Psoriasis	Nickoloff BJ et al., 1991; Biasi et al., 1998

Table 1.2.1 Human diseases in which IL-8 has been implicated

1.3. THE MACROPHAGE

The term 'macrophage' was proposed by Metchnikoff in the late 19th century to describe a large cell, present in connective tissues and fluids throughout the body, with a propensity to phagocytosis (Metchinkoff, 1905). Macrophages are remarkably ubiquitous and have been identified in all animal species studied. The functional repertoire of the macrophage extends beyond phagocytosis to include antigen presentation, anti-bacterial activity, anti-tumour activity and secretion of an array of biologically active peptides and proteins (Nathan et al., 1980; Auger and Ross, 1993; Seljelid and Eskeland, 1993). This supports the view that the macrophage is fundamentally important to the integrity of the multicellular organism.

1.3.1 Origins and tissue distribution of macrophages

The macrophage often exhibits a tissue-specific phenotype, such as the histiocyte in connective tissue, the Kupffer cell in the liver and the alveolar macrophage in the lung. The origin of these cells remained an area of controversy for many years. A series of elegant studies in mice revealed that the resident Kupffer cell (Crofton et al., 1978), alveolar macrophage (Bowden and Adamson, 1980; Blussé van Oud Alblas and van Furth, 1983) and splenic macrophage (van Furth and Diesselhoff-den Dulk, 1984) were all principally derived from the circulating monocyte. It is now accepted that in the steady state, the majority of tissue macrophages derive from the circulating monocyte, itself derived from precursor cells in the bone marrow.

During an acute inflammatory process, neutrophils are the first of the blood leukocytes to be recruited, often within 2 hours of onset. The arrival of circulating monocytes is relatively delayed but, in contrast to neutrophils, monocytes continue to accumulate beyond the first 24 hours (Adams, 1992; Bellingan et al., 1996). Thus, depending upon the stimulus, the monocyte is frequently the predominant inflammatory cell by 48 hours. Once recruited to the inflammatory site, the monocyte differentiates into a macrophage phenotype. Whether recruitment of blood monocytes is the predominant source of increased tissue macrophages during inflammation remains an area of contention.

Although van Furth and colleagues provide compelling evidence that this is indeed the case in a variety of organs (Thomas et al., 1976; Blussé van Oud Alblas et al., 1981; Blussé van Oud Alblas et al., 1983; Diesselhoff-den Dulk and van Furth; 1979), other investigators have demonstrated extensive local proliferation of monocytes and macrophages (De Bakker et al., 1985; Bouwens et al., 1986).

1.3.2 Pulmonary Macrophages

Based on their localisation, it has been proposed that pulmonary macrophages may be characterised into four different types; i) the alveolar macrophage, ii) the interstitial macrophage, iii) the intravascular macrophage and iv) the dendritic cell (Lohmann-Matthes et al., 1994). Alveolar macrophages are located at the interface between air and lung tissue, bathed in surfactant (a protein and phospholipid-rich lining fluid secreted by type II alveolar epithelial cells). In healthy humans and most animals, over 90% of cells retrieved following bronchoalveolar lavage (BAL) are alveolar macrophages (Reynolds, 1987). These mature cells remain prevalent in the lavage fluid after several washes, and since they are the most accessible population of pulmonary macrophages, they are the most studied. There is compelling evidence that the alveolar macrophage predominantly derives from the circulating monocyte (Thomas et al., 1976; Bowden and Adamson, 1980; Blussé van Oud Alblas and van Furth, 1983). The contribution from dividing interstitial cells is relatively small under normal conditions.

1.3.3 Macrophage function in acute inflammation

The macrophage is a functionally complex cell. Many of its multiple roles are manifest in the acute inflammatory response in which, whilst attempting to fulfil a critical role in host defence, the macrophage has the potential to cause tissue injury (Adams and Hamilton, 1992).

1.3.3.1 Macrophage response to bacterial endotoxin

The macrophage plays a major role in the recognition, engulfment and killing of microbial organisms. The response to bacterial invasion is triggered by interaction

between macrophage cell surface receptors and secreted products or bacterial wall components (Noursadeghi and Cohen, 2000), the most important of which is bacterial endotoxin or lipopolysaccharide (LPS). At least 3 cloned macrophage cell surface molecules are known to bind lipid A, the toxic moiety of LPS; CD14, the CD11/18 β2 leukocyte integrin family and the macrophage scavenger receptor (SR). The latter does not possess signalling properties, but is involved in LPS processing and host defence (Haworth R et al., 1997). The CD11/18 integrins are capable of inducing signalling events and macrophage activation (Ingalls and Golenbock, 1995; Ingalls et al., 1997; Ingalls et al., 1998), but the major LPS receptor for macrophages and monocytes is CD14.

1.3.3.2 The CD14 receptor

CD14 is a glycophosphatidylinositol (GPI)-linked protein (Fenton and Golenbock, 1998; Lien et al., 2000 in Update in ITU p164). LPS in the circulation binds lipopolysaccharide-binding protein (LBP) and the resulting complex is able to cross-link CD14 on the cell (Tobias et al., 1993). Soluble CD14 receptor also binds LBP, and this complex can confer endotoxin responsiveness in CD14 deficient cells such as endothelial cells (Tapping and Tobias, 1997). Genetically engineered CD14 deficient mice are completely resistant to endotoxin induced death (Haziot et al., 1996). Deletion of murine LBP greatly diminishes LPS responsiveness *ex-vivo*, but the *in-vivo* effects of endotoxaemia are similar to wild-type mice (Jack et al., 1997; Wurfel et al., 1997). LBP is an acute phase protein and is likely to play a complex role in host defence; its function depends on its concentration which in turn varies in response to systemic inflammation (Lien et al., 2000).

CD14 protein lacks transmembrane and intracellular domains and contains no known signalling motifs. It has subsequently been demonstrated that CD14 acts by associating with a distinct transmembrane signal transducing protein of the Toll family (Yang et al., 1998; Kirschning et al., 1998). The Toll and Toll-related proteins are a widely conserved family of innate immune recognition receptors. To date at least 8 mammalian Toll-like receptors (TLR) have been recognised (reviewed in Means et al., 2000). It was initially believed that TLR2 was the primary signalling receptor in human cells, but this

observation may require reinterpretation following the report that commercial preparations of LPS typically contain highly bioreactive contaminants generically mis-termed endotoxin proteins (Hirschfeld et al., 2000).

1.3.4 Macrophage and inflammatory cytokines

The cytokine superfamily of low molecular weight proteins includes interleukins, growth factors, colony stimulating factors, tumour necrosis factor (TNF) and chemokines. The tremendous range of activity demonstrated by these proteins implicates cytokines as having potential roles in the initiation, maintenance and resolution of inflammation. Depending upon state of activation and stimulus, the macrophage has the capacity to generate a plethora of cytokines and express complementary receptors, demonstrating potential autocrine regulation. The cytokines TNF-α, IL-1, IL-6 are discussed in **Chapters 1.1.5** and the chemokine IL-8 is discussed in **Chapter 1.2.3**.

1.3.5. Role of the macrophage in inflammatory diseases

The proposition that the macrophage plays a central role in the inflammatory process is supported by studies in macrophage-depleted animals. Several techniques for selective macrophage depletion, including delivery of liposome-encapsulated toxic agents that are preferentially taken up by macrophages, have been described (van Rooijen et al., 1997). Macrophage depletion studies have highlighted the double-edged nature of macrophages in both host defence and in tissue injury. Acute inflammation in the lung (Sone et al., 1999; Ofulue and Ko, 1999; Zhang-Hoover et al., 2000), kidney (D'Souza et al., 1999), joints (Richards et al., 1999, Barrera et al., 2000), brain (Zito et al., 2001) has been shown to be attenuated by selective macrophage depletion. In contrast, studies of endotoxaemia, for example, have revealed enhanced tissue injury and lethality following macrophage depletion (Groeneveld et al., 1988; Nieuwenhuijzen et al., 1993).

1.3.6 Macrophage studies *in vitro*

In vitro studies of human macrophages are limited by the relatively difficulties in efficiently extracting tissue macrophages. Whilst alveolar macrophages are relatively accessible, bronchoalveolar lavage still represents an invasive procedure. Peripheral blood monocytes are in contrast relatively accessible. In the presence of serum, adherent cultured monocytes undergo maturation changes consistent with differentiation into macrophages (Johnson et al., 1977)

Since the macrophage is intimately associated with the inflammatory process, studying its adaptation and response to the local micro-environment is a salient area of research. Hyperthermia, acidosis and glucose deprivation are all relevant to inflamed tissue. Tissue oxygenation may however be particularly relevant to a variety of human diseases.

1.4.TISSUE OXYGENATION

1.4.1. Oxygenation in Health

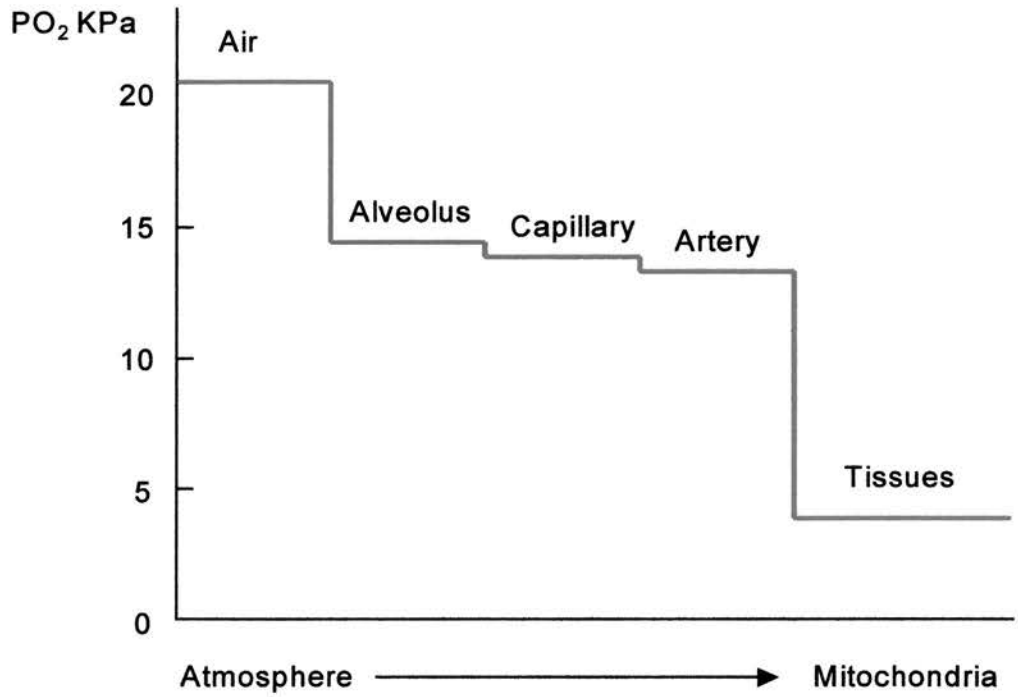
1.4.1.2. Ventilation, perfusion and gas exchange

The percentage of oxygen in inspired air (FiO_2) is constant at 21%. At normal atmospheric pressure the PO_2 of dry air is 21.3 KPa. However as oxygen is transported into the lungs through to the alveolar capillary interface the PO_2 progressively falls. These initial step-wise reductions in PO_2 (**Figure 1.4.1**) represent the effects of humidification, and more importantly, the effect of constant oxygen uptake and of CO_2 addition. Hence, the 'normal' alveolar PO_2 is approximately 13.6 KPa. This figure represents a mean value, as it should be emphasised that even in a healthy lung there are regions of comparatively high and low ventilation and high and low perfusion. This implies significant ventilation / perfusion (V/Q) mismatch resulting in alveolar units that are relatively hypoxic, and hyperoxic compared to the mean. Although actual measurements of PO_2 within individual units is not technically feasible, indirect methods have shown that the mean alveolar PO_2 is greater than 17.5 KPa near the lung apex but less than 12 KPa at the base (West, 1985). This V/Q mismatch is the major cause of a further stepwise reduction in PO_2, such that the mean systemic arterial blood PO_2 is around 11.5 KPa. The diffusion of oxygen across the alveolar / capillary membrane is a passive process. However since the diffusion distance is small (<0.5 μM) and the diffusion gradient high (8 KPa on average), oxygen floods into the bloodstream. Equilibrium between capillary and alveolar oxygen is attained within 0.25 seconds of the average 0.75 seconds that a red blood cell is in transit through the gas exchange portion of a capillary.

1.4.1.3. Oxygen Transport in Blood

Oxygen is principally transported bound to haemoglobin (Hb), a complex four polypeptide protein. Oxygen dissolved in plasma when breathing 21 % oxygen is normally negligible compared to that carried by haemoglobin. One gram of Hb is able to carry 1.39 ml of oxygen if 100 % saturated. The binding of Hb to oxygen is such that

Figure 1.4.1



Legend for Figure 1.4.1 Oxygen tension from atmosphere to mitochondria.
There is a progressive reduction in PO2 from air to the to site of oxidative phosphorylation, the mitochondria.

even when exposed to relatively low oxygen tensions, Hb is over 95% oxygen-saturated. It is only as PO_2 falls below 7.5 KPa that Hb becomes rapidly desaturated, such that P_{50} (defined as the oxygen concentration at which 50 % of the Hb is saturated) is 3.5 KPa. Oxygen content of the blood can be calculated as follows:

$$\text{Oxygen content} = 1.39 \times \text{Hb concentration} \times \text{percentage saturation} + \underbrace{0.003 \times PO_2}_{O_2 \text{ dissolved in plasma}}$$

Hence a healthy individual with normal Hb level of 15 g / 100 ml blood, is potentially able to carry 20.8 ml oxygen per 100 ml of blood (1.39 x 15 x 1 + [0.0003 x 11.5]).

1.4.1.4. Tissue Oxygenation

The primary site of oxygen utilisation is at the tissue level, wherein the generation of high-energy phosphate bonds (ATP) via the mitochondrial electron transport chain is

fundamental to normal cell function and survival. Constant oxygen utilisation by tissues results in a gradient favouring release of oxygen from Hb. This chemical release is further enhanced by the effects of raised temperature, increased PCO_2 and lowered pH and increased 2,3 diphosphoglycerate concentration, all features of metabolically active tissue. The released oxygen passively diffuses through the endothelium and surrounding tissue to mitochondria. Potential diffusion distances vary between tissues. In the left ventricle, each capillary serves a cellular zone of approximately 12.5 μm. The corresponding figure is 20 μm in the brain and 200 μm in skeletal muscle. In poorly vascularised tissue such as fat and cartilage diffusion distances are greater still. Hence, the PO_2 at the mitochondrial level is considered to be 0.7-1.3 KPa. (Gayeski and Honig, 1986; Leach and Treacher 1998). Although incompletely understood, perfusion and diffusion distances are part of the explanation for the observation that in healthy resting adults only about 25 % of delivered oxygen is extracted by tissues.

1.4.1.5. Mitochondria – the site of oxidative phosphorylation

The mitochondrial electron transport chain (**Figure 1.5.2**) is the site of ATP production under normal conditions. Virtually all cellular processes are driven by the energy released when ATP undergoes hydrolysis to form adenosine diphosphate (ADP) and inorganic phosphate (Pi). The generation of ATP occurs through a series of redox reactions, in which electrons are transferred step-wise from nicotine adenine dinucleotide (NADH) and flavin adenonine dinucleotide ($FADH_2$), to the terminal electron quencher, oxygen. The transfer occurs through a series of intermediate complexes (I, III and IV) of progressively lower reducing potential. Each complex contains redox-active electron carriers, several of which are located in the inner mitochondrial membrane. As each electron is accepted, the energy released is used to actively pump protons (H^+) from the miochondrial matrix into the intermembrane space. An electrochemical gradient is thus created across the inner membrane. The presence of this gradient drives hydrogen ions through a mitochondrial enzyme, the F_0F_1ATPase, that in turn catalyse the formation of ATP from ADP and Pi.

1.4.2. Causes of hypoxia

In 1920, Barcroft proposed a form of classification for the causes of hypoxia. With minor modifications over time, this classification is still widely applied: (1) hypoxic hypoxia, in which the PO_2 of the arterial blood is reduced (termed hypoxaemia); (2) anaemic hypoxia, in which arterial PO_2 is normal but reduced haemoglobin lowers oxygen carrying capacity in blood; (3) ischaemic or stagnant hypoxia, a consequence of impaired blood flow to tissues; (4) histotoxic or cytopathic hypoxia, in which despite adequate oxygen delivery, the tissue is unable to take up or utilise oxygen.

1.4.2.1. Hypoxic Hypoxia

Maintenance of adequate systemic arterial PO_2 is dependent upon normal pulmonary function. Physiologically, there are five primary mechanisms that may result in hypoxaemia (a reduction in arterial PO_2):

i. A reduction in inspiratory oxygen fraction (FiO_2). This is most commonly a consequence of increasing altitude. Atmospheric pressure decreases with distance above sea-level in an approximately exponential manner. The pressure at 5500 meters is 50% of the sea-level value, resulting in a FiO_2 of approximately 10 KPa. This results in a corresponding fall in alveolar PO_2 to around 5.5 KPa and as a consequence the 'driving' pressure gradient across the alveolar / capillary membrane is reduced leading to hypoxaemia.

ii. Hypoventilation. Alveolar PO_2 is a balance between rate of oxygen removal by blood and replenishment through alveolar ventilation. Hypoventilation secondary to reduced central drive to breath, weakness of respiratory muscles or injury / deformity of the thoracic cage results in reduced alveolar PO_2 (and increased PCO_2) and consequently arterial hypoxaemia.

iii. Diffusion impairment. As previously described, the diffusion of oxygen across from alveolus to red blood cell is rapid and efficient in healthy lungs. In theory diseases in

which the alveolar / capillary membrane is thickened, such as in idiopathic pulmonary fibrosis, result in hypoxia secondary to impaired diffusion. In reality V/Q mismatch and shunt are likely to make a greater contribution to hypoxia than diffusion impairment in such diseases.

iv. Shunt. This refers to deoxygenated blood entering the systemic circulation without passing through ventilated areas of lung. In normal health, a proportion of deoxygenated bronchial artery and coronary venous blood is shunted directly into the pulmonary veins and left ventricle respectively. This has a near-negligible effect on arterial PO_2 and is often termed anatomical shunt, or more appropriately, venous admixture. In diseased lungs in which, for example, there is atelectasis (alveolar collapse) and consolidation, as occurs in ARDS, shunting is an important contributor to hypoxaemia.

v. V/Q mismatch. As described previously, a normal lung exhibits areas in which ventilation and perfusion are not equally matched, and is the main reason arterial PO2 is usually slightly lower than average alveolar PO2. Exaggerated V/Q mismatch is probably the most important contributor to hypoxia in most primary lung diseases including chronic obstructive pulmonary disease (COPD), diffuse parenchymal lung disease and ARDS.

1.4.2.2. Anaemic hypoxia.

A healthy individual with normal Hb level of 15 g / 100 ml blood, is potentially able to carry 20.8 ml oxygen per 100 ml of blood. An anaemic patient with a Hb of 10 g / 100 ml, assuming normal gas exchange, will display a normal arterial PO_2, since the affinity of each Hb molecule to oxygen remains high and gas exchange is unaffected. However the total oxygen carrying capacity of the blood is reduced by 1/3 to around 13 ml oxygen per 100 ml blood, potentially leading to tissue hypoxia.

1.4.2.3. Stagnant or ischaemic hypoxia

This represents a spectrum of pathology from insufficient circulation or perfusion, as may occur during periods of systemic hypotension, through to complete blood stasis, as occurs in vascular occlusion. The tissue response to ischaemic hypoxia represents not just the effects of oxygen deprivation but also the effects of accumulation of waste products. Blood flow in the centre of a wound is reduced due to thrombosis in the microcirculation.

1.4.2.4. Histotoxic or cytopathic hypoxia

This term refers to impaired cellular utilisation of oxygen such that mitochondrial ATP generation is reduced despite adequate oxygen supply. This includes exposure to specific toxins and genetic defects in cellular respiration. Cyanide, sodium azide and rotenone are potent inhibitors of the mitochondrial electron transport chain and in that respect mimic severe hypoxia. A number of rare neurological or systemic diseases are caused by mitochondrial DNA mutations impairing oxidative phosphorylation (reviewed by Johns, 1995). In clinical practice however, it is becoming clear that impaired oxygen utilisation is not restricted to the models described. In patients with severe sepsis, tissue oxygen uptake is thought to be impaired. Endotoxin, the lipopolysaccahride (LPS) component of the outer membrane of Gram-negative organisms, is implicated in the pathogenesis of sepsis and has been shown to impair oxygen utilisation *in vitro* (James et al., 1995; Motterlini et al., 1998). Studies have demonstrated that this impaired utilisation occurs at the mitochondrial level (Bankey et al., 1994; Zingarelli et al., 1997), though the precise site of action within the electron transport chain is debated (Fink, 2000).

1.4.3. Methods for measuring cellular and tissue PO_2

An accurate measurement of oxygen tension at a cellular level is a desirable aim for experimental studies and in the clinical arena. Direct measures of PO_2 would seem desirable, but in the clinical settings, an indirect measure is often more practical.

1.4.3.1. Polagraphic electrode and optical probes

A commonly used method for directly measuring tissue oxygenation is through polagraphic electrode and more recently optical probes. Polagraphic probes (e.g. the Clarke electrode) comprise a platinum electrode and a silver counter / reference electrode inside a stainless steal sleeve. A gas permeable membrane fits over the sleeve and allows oxygen to diffuse through it, whereupon it is reduced at the platinum electrode. The magnitude of the electrical current generated is determined by the rate of diffusion to the electrode, which is proportional to the partial pressure of oxygen outside the membrane. Such probes have been used to measure tissue PO_2 in clinical and animal studies (Dewhirst et al., 2000; Yeh et al., 1995). Technological advances have resulted in electrode tips sufficiently small to measure intracellular levels of oxygen (Lau et al., 1992). The efficacy and reproducibility of one particular system (Eppendorf) has been demonstrated in a number of studies (reviewed in Dewhirst et al., 2000). However, there are concerns that in biological systems, interference from tissue ions may disrupt miroelectrode measurements.

Fibre optic probes have also recently been developed to measure tissue oxygenation directly (Collingridge et al., 1997). A fluorescent or phosphorescent dye (e.g. a porphyrin) is placed on the tip of a glass fibre which is clad in an oxygen permeable membrane. The dye is illuminated with blue light from a laser and the lifetime (quenching) of the resultant fluorescent signal is inversely proportional to the amount of molecular oxygen in the tissue. As with electrode probes, the membrane may be susceptible to fouling in the presence of proteins. In addition, neither the polagraphic nor optic probe methods would be suitable for measuring PO_2 in multiple cells or multiple sites simultaneously.

1.4.3.2 Near infrared spectroscopy (NIRS)

Non-invasive methods, such as NIRS represent a potentially useful method for measuring tissue oxygenation (Jobsis, 1977). The oxygenation status of haemoglobin, myoglobin and cytochrome oxidase alters the wavelength and intensity of absorption of near infrared

light, which may be measured spectrophotometrically. NIRS has been used to assess hepatic (El-Desoky et al., 2001), cerebral (Hayakawa et al., 1996; Madsen and Secher, 1999), cardiac (Van Beek et al., 1996), lung (Okimasa et al., 2001) and skeletal muscle (McKinley et al., 2000) tissue oxygenation. However, absorption signals from ischaemic tissue is not often homogenous, and the technique does not distinguish oxygenation in circulating blood from that in tissue.

1.4.3.3. Direct chemical and biological markers of hypoxia

Recently, the phosphorescence quenching principle described for optic probes (**1.4.3.1**) has been adapted to achieve non-invasive measurement of tissue PO_2. The quenching in rat skeletal muscle of a phosphorescent dye injected intravenously was measured by laser microscope (Shibata et al., 2001). This technique requires sophisticated hardware. A number of investigators have reported the use of specific drugs that selectively bind to hypoxic cells as a means of identifying hypoxic cells in tumours (Evans et al., 2000; Raleigh et al., 1996) in sepsis models (Hotchkiss et al., 1991) and in lung injury (Vujaskovic et al., 2001). These drugs are generally 2-nitroimadazole compounds and are lipophilic and freely diffusible, readily traversing cell membranes (reviewed in Hodgkiss, 1998). The drugs are metabolised (reduction of the nitro-group) in the presence of oxygen. In hypoxia, bioreduction does not occur and the drug is converted to highly reactive free radical molecules that bind to proteins and DNA. The drug metabolites may be detected in tissue sections with anti-nitroimadazole antibodies (Raleigh et al., 1996, Arteel et al., 1998). Alternatively, if the drug is pre-labelled with an isotope, hypoxic tissue may be identified by positron emission tomography (PET) and related imaging modalities.

A limitation of chemical markers of hypoxia is that quantifying tissue oxygenation is difficult. There is evidence that as oxygen concentration falls from ~3-10 %, drug binding progressively increases to reach a maximum under anoxic conditions (Rasey et al., 1987; Rasey et al., 1989). However, bioreductive drugs may be more reliable as markers of presence or absence of hypoxia.

The 2-nitroimadazole compounds appear to be non-toxic and have been used to study to tumour hypoxia in patients. However, an attractive proposition may be the detection of a naturally occurring marker of cellular hypoxia. Hypoxia-inducible factor-1α (discussed in detail in **Chapter 1.5.**) is a novel basic helix-loop-helix protein, that *in vitro* cell culture is thought only to be expressed at $PO_2 < 5KPa$ (Huang et al., 1996). In keeping with this is the finding that in range of normal tissues obtained at biopsy, HIF-1α expression was negligible, compared to significant expression in a variety of tumour biopsy specimens (Talks et al., 2000). In contrast however, Stroka et al., 2001 have demonstrated basal HIF-1α expression in brain, kidney, liver, heart and skeletal muscle of mice under normoxic conditions, with upregulation following hypoxia. Hence, the premise that HIF-1α may only be expressed under hypoxic conditions may not be valid. Indeed, there is a growing body of evidence that non-hypoxic pathways may induce HIF-1α expression (Hellwig-Burgel et al., 1999; Feldser et al., 1999; Haddad and Land, 2001).

1.4.3.4. Markers of oxygen metabolites

Rather than direct measures of molecular oxygen, detection of an oxygen metabolites such as reactive oxygen species (ROS) have been studied in cell culture systems. A variety of fluorescent probes are available (Molecular Probes, Eugene, OR) that in the presence of ROS display a detectable change in fluorescence. Such probes have been used in studies of hypoxic cells in culture (Vanden Hoek et al., 1998; Asiedu et al., 2001). However, the precise effect of hypoxia on ROS generation is an area of considerable debate (**Chapter 1.5.5**), and studies with fluorescent probes demand the use of stringent controls.

The direct measures of hypoxia dicussed thus far are largely experimental or require further validation before routine use in clinical practice. In general, measures of tissue hypoxia in the clinical setting tend be indirect, and these are discussed in the context of the critically ill patient.

1.4.4. Hypoxia in the critically ill patient

'Critical illness' in the context of this thesis is a term that encompasses acute medical conditions, which have in common a requirement for early medical intervention, close monitoring and carry a significant risk of death. Such patients are optimally managed in the setting of an intensive care unit (ICU). The spectrum of disease is wide, but sepsis, trauma, haemorrhage, aspiration injury and pancreatitis are common precipitants for ICU admission. A characteristic feature of severe disease of this type is the development of SIRS (**Figure 1.1**) which may in turn progress to MODS and ARDS.

Systemic and tissue hypoxia is of particular relevance in patients with critical illness. It is apparent that the spectrum of disease in such patients not only causes hypoxia through a variety of mechanisms, but also that hypoxia may itself contribute to the progression of disease and development of widespread tissue injury and organ failure.

1.4.4.1 Causes of hypoxia in the critically ill

A number of factors contribute to systemic and tissue hypoxia in critically ill patients. In patients with major trauma, haemorrhage and hypotension are often self evident, as is pulmonary shunting in a patient with lobar pneumonia. A significant proportion of patients develop impaired gas exchange secondary to acute lung injury and ARDS. More subtle causes of tissue hypoxia are believed to occur at a microvascular level in patients with SIRS. In such patients, particularly if associated with sepsis, there is evidence of altered distribution of blood supply between organs (Viires et al., 1982; Lang et al., 1984; Fong et al., 1990; Duranteau et al., 1996), within organs (Carlton et al., 1976; Ratcliffe et al., 1986; Kreimeier et al., 1990; Drazenovic et al., 1992), and reduced oxygen uptake (Bredle et al., 1989; Simonsen et al., 1994; Fink, 2000). These factors all contribute to extreme tissue oxygen deprivation (Uusaro and Russell, 1999; Evans and Smithies, 1999).

1.4.4.2. Hypoxia as a cause of tissue injury and MODS

Whilst it is generally accepted that the development of MODS is multifactorial (Bone, 1996) there is evidence that tissue hypoxia is itself a significant contributor to the pathophysiology of organ failure. The profound effects of hypoxia on normal homeostasis in the *in vitro* and animal studies make this an attractive proposition. Evidence from human studies is more circumspect. This reflects the difficulties associated with measuring tissue oxygenation and correlating this with outcome in a complex disease. The relatively few prospective studies to date that have attempted to investigate this have used indirect measures of tissue oxygenation. Accepting the inherent flaws in such methods, the weight of evidence favours an association between the degree of tissue hypoxia and development of MODS.

Oxygen debt is a reasonable measure of tissue hypoxia. However, there have been no prospective studies that have directly investigated an association between oxygen debt and MODS. In a small number of studies, interpretation of the available data does allow this measure to be made. In post-operative patients, those who maintained higher levels of oxygen delivery (DO_2) and oxygen consumption (VO_2) and hence lower oxygen debt, showed a reduction in the progression to multiple organ failure and a 4-5 fold reduction in mortality (Hayes et al., 1994; Brealey and Singer, 2000). In a study of different ventilation strategies in ARDS, patients with high oxygen debt had excess mortality and MODS (Spec-Marn et al., 1993; Uusaro and Russell, 1999).

Blood lactate is an indirect measure of anaerobic respiration, though factors other than tissue hypoxia may influence lactate metabolism (James et al., 1999; Gladden, 2001). In general however, there is a direct relationship between elevated lactate concentration and mortality in the critically ill (Broder and Weil, 1964; Vitek and Cowley, 1971; Bakker et al., 1991) The relationship between lactate and development of MODS appears to be strongest in patients with multiple trauma and haemorrhage (Roumen et al., 1993; Sauaia et al., 1994; Moore et al., 1992; Manikis et al., 1995).

It has long been recognised that splanchnic hypoxia may be associated with the development of organ failure (Rowell et al., 1968; Price et al., 1966). Several studies have identified an association between reduced gastric mucosal pH, a measure of splanchnic hypoxia, and the development of MODS following trauma (Miller et al., 1996), sepsis (Marik, 1993) and following major surgery (Doglio et al., 1991; Mythen and Webb, 1994).

Hypoxia is clearly detrimental to health. As such, a fundamental therapeutic target in the critically ill patients is to improve tissue oxygenation, primarily through increased oxygen supply. Oxygen however is a toxic molecule, and hyperoxia itself an important cause of tissue injury.

1.4.5. Hyperoxia and disease

Joseph Priestly, the discoverer of the O_2 molecule in 1774, not only recognised it as "an air five or six times as good as common air", but also foresaw its "adverse effects on the healthy state of the body" (cited in Partington, 1989).

In evolutionary terms, it has been suggested that the diversification of multicellular organisms has in part been an adaptive response to the progressive rise in atmospheric oxygen from negligible levels to its current level over millions of years (Knoll, 1996). Adaptation has harnessed the potential for molecular oxygen to be converted to energy, whilst developing host defences to protect against toxic oxygen metabolites (Shanklin, 1969; Fridovich, 1978). The rapid acclimatisation of the new-born from an intrauterine PO_2 of 3-4 KPa to one of 20 KPa within the first few postnatal breaths is testament to the efficiency of these host defences. This ontogenic event represents an innate response to relative hyperoxia. In human disease, true hyperoxia is invariably a consequence of therapeutic delivery of oxygen, and as such hyperoxic tissue injury is a phenomenon of the modern age.

The brain and the neonatal retina are susceptible to hyperoxic injury (Hunter and Mukai, 1992). However, the lung is often exposed to the highest concentrations of oxygen and is

particularly susceptible to its toxic effects. Much of the damage to cellular DNA, lipids and proteins is believed to be mediated by reactive oxygen species (ROS), which include superoxide anion (O_2^-) hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^·$) and peroxynitrite ($ONOO^-$) (Thannickal and Fanburg, 2000; O'Reilly, 2001).

In adult lungs, delivery of 100% oxygen results in characteristic progressive morphological changes in the lung (Crapo JD, 1986). Within the first 24 hours of exposure there is little evidence of inflammatory injury. Atelectasis caused by lack of unabsorbed alveolar nitrogen is evident (Wagner et al., 1974) but these and any other functional abnormalities of the lower respiratory tract are transient (Sackner et al., 1975; Singer et al., 1970). Davis et al., (1983) showed healthy humans exposed to 17 hours of 100% oxygen developed mild but significant lung leak, as measured by protein in BAL fluid, without inflammatory cell influx. Exposure to these concentrations beyond two to three days leads to alveolar-capillary wall oedema, neutrophil infiltration, lung protein leak and hyaline membrane formation; broadly the pathological features of ARDS (Hasleton, 1981; Sevitt, 1974; Katzenstein, 1976). It is apparent that prolonged exposure to 100% O_2 usually occurs in the context of a ventilated patient with existing parenchymal lung injury. As such, the toxic effects of oxygen may not be distinguishable from the underlying disease or from the effects of ventilation (Carvalho et al., 1998). However, the association between oxygen delivery and bronchopulmonary dysplasia (BPD) in non-ventilated neonates supports the notion that 100% oxygen induces lung injury. At inspiratory concentrations of $\leq$50% oxygen toxicity, even after prolonged exposure, is unlikely to occur (Hayatdavoudi et al., 1981).

1.5. THE CELLUAR RESPONSE TO HYPOXIA

Cellular responses to hypoxia appear to be a universal phenomenon; all mammalian cells and, it is speculated, all bacteria, yeast, invertebrates and vertebrates are able to sense and respond to hypoxia (Semenza, 1999). The cellular response to hypoxia is determined by: 1) the individual cell phenotype, 2) the degree of oxygen deprivation (anoxia versus hypoxia) and the period of oxygen deprivation (acute or chronic).

1.5.1. Cell Phenotype and hypoxic response

Whilst all mammalian cells display oxygen-sensing capabilities, some have evolved specialist oxygen sensing roles. Cells in the carotid body, airway neuroepithelial bodies and pulmonary arteries mediate homeostatic cardiovascular and pulmonary responses to a reduction in arterial or alveolar PO_2. A reduction in arterial PO_2 is sensed by the glomus type 1 cells of the carotid body. This triggers release of transmitters that determine afferent nerve discharge, stimulating the brain stem to increase ventilation (McDonald, 1981; Donnelly, 1996). Membrane depolarisation secondary to inhibition in K^+ channel conductance is a charactersitic feature of the hypoxic response in these cells (Lopez-Barneo et al., 1988). The precise signal transduction pathway from hypoxic sensing to change in K^+ channel activity remains elusive. It has been demonstrated that the sensing mechanism in these specialist cells may be independent of intracellular events, since even excised patches of membrane retain an O_2 sensing function (reviewed in Haddad and Jiang, 1997). However there is also evidence that many of the proposed sensing mechanisms discussed in **Chapter 1.5.5** are likely to be applicable to specialist and non-specialist cells alike.

1.5.2. Anoxia versus hypoxia

Although there is considerable overlap, the cellular response to anoxia differs from its response to relative hypoxia. Sustained anoxia inevitably leads to cell death; hypoxia, whilst potentially lethal, may induce a range of adaptive responses (summarised in **Figure 1.5.1**).

Although the nomenclature in the literature is inconsistent, here anoxia refers to a state in which diffusion of oxygen to the mitochondria falls to below the level required to sustain oxidative phosphorylation (the critical PO_2 or P_{crit}) and hypoxia refers to a state in which oxidative phosphorylation and ATP generation is impaired, though not necessarily abolished.

1.5.2.1. Bioenergetic response to anoxia

In anoxia, the demand for ATP can be met only through anaerobic glycolysis (the 'Pasteur effect'). This offers limited respite. In most cells, glycolysis can maximally generate less than 20% of the ATP produced through aerobic mitochondrial phosphorylation. The stores of fermentable substrate are limited and end-products, primarily lactic acid, are toxic. As ATP declines therefore, there is failure of the ion-motive ATPases, followed by membrane depolarisation and uncontrolled influx of Ca^{2+}. The rise in free cytosolic Ca^{2+} activates Ca^{2+}- dependent phospholipases and proteases, hastening further membrane depolarisation and consequently cell swelling and death through necrosis (Hochachka, 1986). The time of survival is to some extent dependent upon cell type. Animal studies have revealed that neuroglial cells lose physiological function within 1 minute, whereas skeletal muscle remains viable for several hours (Jones, 1996). *In vitro*, some cell-types are able to adapt to anoxia through 'metabolic depression'- downregulation of selected ATP-utilising systems, and this may account for the differing survival times of anoxic tissues. Hepatocytes for example have significant capacity to arrest non-essential protein synthesis, channelling the energy spared to critical membrane ion transport activity (Buc-Calderon et al., 1993).

1.5.2.2. Bioenergetic response to hypoxia

In contrast to anoxia, most mammalian cells exhibit a range of adaptive responses to sustainable levels of hypoxia (**Figure 1.5.1**). At the mitochondrial level, oxygen supply is insufficient to maintain maximal ATP generation. There is a change in the reducing capacity of the electron transport chain, resulting in channelling of some of the energy reserved to maintain the proton-motive force to the generation of ATP in the

mitochondrial matrix (Jones, 1996). Studies have revealed that in some cells a degree of metabolic depression can occur. Cardiac myocytes are able to suppress metabolic rate by up to 50% without loss of viability (Budinger et al., 1998). This phenomenon does not imply that hypoxia is without repercussion for cell survival. Not all cells are able to display such bioenergetic adaptive response, and even in those that do, the dramatic reduction in ATP will impair ion-motive ATPases. Glycolysis is stimulated at levels above P_{crit} and even small reductions in pH can cause lysosomal rupture and release of degredative enzymes into the cytoplasm (Ichihara et al., 1991). Uncoupled electron flow through the mitochondrial chain can result in toxic ROS generation. These mechanisms may lead to cell death by necrosis, identical to anoxia-induced death but at a slower rate. Recently however it has become apparent that in addition to necrosis, hypoxia can induce death through apoptosis.

1.5.3. Hypoxia and apoptosis

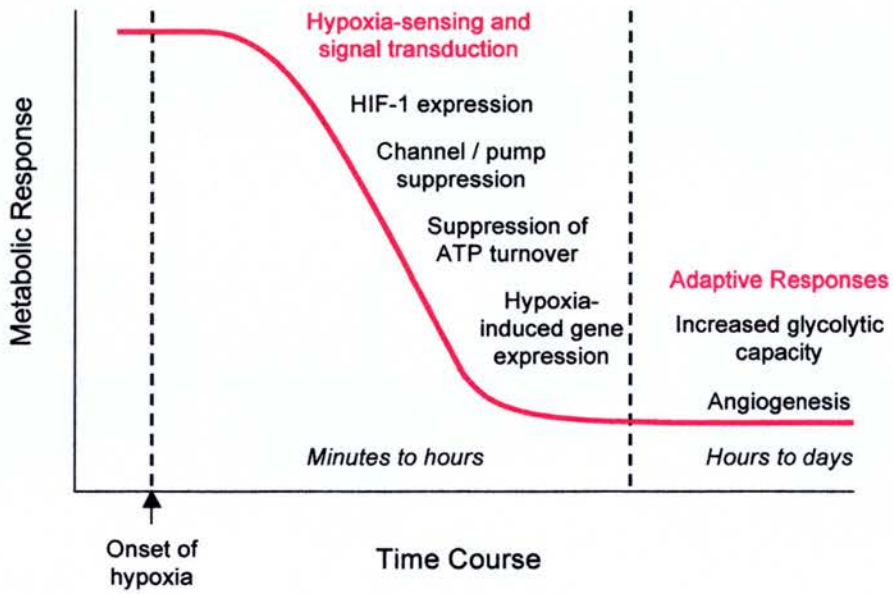
Apoptosis, the phenomenon of programmed cell death, is classically characterised by cell shrinkage, nuclear condensation and pronounced membrane blebbing (Wylie, 1981). Hypoxia (> 0% O_2) has been shown to induce apoptosis in a variety of tumour cell lines, including HT29 adenocarcinoma cells (Yao et al., 1995), MCF-7 breast cancer cells (Amellem et al., 1997) and PC-12 cells (Tsujimoto et al., 1997). Similarly, primary cultures of sympathetic neurons (Rosenbaum et al., 1994) and hepatocytes (Shimizu et al., 1996) have been shown to undergo apoptosis in hypoxic conditions. However, the effect of hypoxia on cell survival appears to be more complex than might be surmised from these data. In thymocytes, rapid induction of hypoxia (0.1% O_2) protects against dexamethasone-induced apoptosis but prolonged exposure to severe hypoxia increased spontaneous apoptotic cell death (Stefanelli et al., 1995). Recent studies have suggested the presence of O_2-dependent and O_2-independent apoptotic pathways in thymocytes (Torres-Roca et al., 2000).

Myeloid cells, at least in culture, may be particularly tolerant to oxygen deprivation. Human monocyte-derived macrophages were found not only to be viable for up to 48 hours in a 2% oxygen atmosphere, but also to increase expression of ORP150 (oxygen-

regulated protein 150 kDa), a novel protein which may convey protection from apoptosis (Tsukamoto et al., 1996). Extended cell survival has also been described in hypoxic compared to normoxic neutrophils *in vitro* (Hannah et al., 1995).

The signalling events that determine cell survival, death through apoptosis or death through necrosis following oxygen deprivation are elusive. The modulation of intracellular ROS may be relevant, but pharmacological manipulation of ROS does not necessarily mimic the effects of hypoxia (Torres-Roca et al., 2000). It has been proposed that intracellular ATP levels are a determinant of the mode of cell death; if ATP is depleted then death occurs through necrosis, whereas ATP availability favours apoptosis (Leist et al., 1997; Eguchi et al., 1997).

Figure 1.5.1



Legend for Figure 1.5.1. The cellular metabolic and adaptive responses to hypoxia over time. A series of cellular events are initiated within minutes of onset of hypoxia. These events are designed to both reduce metabolic demand and to initiate adaptive response required for cell survival. Adapted from Boutilier and St-Pierre, 2000, with the kind permission of the authors.

1.5.4. Hypoxic gene regulation

It has long been recognised that changes in gene expression are a consistent feature amongst the adaptive responses to hypoxia,. Our understanding of the mechanisms by

which this occurs have recently been greatly advanced by studies in the hypoxic regulation of erythropoeitin and the subsequent discovery of hypoxia inducible factor-1 (HIF-1)

1.5.4.1. Erythropoeitin and the discovery of HIF-1

Erythropoeitin, a glycoprotein hormone required for the proliferation and differentiation of erythroid cells (reviewed in Jelkmann, 1992), is produced in peritubular interstitial renal and specialised hepatic cells. The finding that EPO gene transcription also occurred in Hep3B and HepG2 human hepatoma cell lines (Goldberg et al., 1987) allowed investigators to study its transcriptional regulation. A region in the 3'-flanking region of the gene which appeared essential for hypoxic induction of EPO synthesis (Beck et al., 1991; Pugh et al., 1991; Semenza et al., 1991) was identified. This 50-nucleotide (nt) sequence, designated a hypoxia responsive element (HRE), was found to bind to a protein (HIF-1) from nuclear extracts from hypoxic, but not normoxic Hep3B cells (Semenza and Wang, 1992).

The hypoxic inducibilty of HIF-1 is found to be generalised, occurring in all cell types tested (Wang and Semenza, 1993). Consistent with this was the finding that transient transfection of the 50-nt element, coupled to a gene other than EPO (e.g. alpha-globin), resulted in hypoxic inducibility of the coupled gene in a wide variety of cell lines (Maxwell et al., 1993). These data strongly supported the hypothesis that much of the machinery for hypoxia sensing and signal transduction is shared between different cell types.

1.5.4.2. HIF-1 protein characterisation

Biochemical purification and DNA affinity chromatography has revealed that HIF-1 was a heterodimer consisting of HIF-1α (120 kDa, 826 amino acids) and HIF-1β (91/94 kDa, 774/789 amino acids) subunits. Both subunits contact DNA in the major groove at a site containing the core sequence 5'-BRCGTGSK-3', where B is C or G or T, S is C or G, K is G or T and R is A or G (Wang and Semenza, 1995). Subsequent protein microsequence

analysis and cDNA cloning has revealed that both proteins contain a basic helix-loop helix (bHLH) motif which is common to many transcription factors. This domain mediates protein dimerisation through juxtapostion of basic amino acids from each monomer subunit to form a functional DNA-binding domain. HIF-1 belongs to a subset of bHLH proteins that require a second domain for efficient dimerisation. This domain is termed the PAS domain [an acronym derived from Period (PER), aryl hydrocarbon receptor nuclear transporter (ARNT), and single-minded (SIM)] and, as with the bHLH domain is sited at the amino (N) terminal half of both HIF-1α and HIF-1β.

Analysis of HIF-1β revealed this protein to be identical to ARNT, a ligand-activated transcription factor involved in cellular responses to xenobiotic agents (reviewed in Hankinson, 1995). HIF-1α, in contrast, was identified as a novel bHLH-PAS protein. Cloning experiments have greatly expanded the number of recognised bHLH-PAS proteins and recently a protein which shows 48% sequence homology to HIF-1α has been identified by several groups independently and reported as endothelial PAS protein-1 (EPAS-1) (Tian et al., 1997), HIF-like factor (HLF) (Ema et al., 1997), member of PAS superfamily 2 (MOP2) (Hogenesch et al., 1997) and HIF-related factor (HRF) (Flamme et al., 1997). EPAS-1, like HIF-1α, is hypoxia-inducible and can dimerise with HIF-1β and bind to similar DNA elements. Its role in gene transcription has not been as well studied as that for HIF-1α.

1.5.4.3. Regulation of HIF-1α protein

ARNT protein is constitutively present in both cell cytoplasm and nuclei under normoxic conditions. Hypoxia induces a moderate rise in nuclear β-subunit levels. By contrast HIF-1α protein is virtually absent in normoxic cells, but can be detected in nuclei within minutes of hypoxic induction, reaching peak levels after 4-8 hours of continuous hypoxia and thereafter gradually falling (Wang et al., 1995A). The activity of HIF-1 is thought therefore to be governed principally by the α-subunit. Under hypoxic conditions ($PO_2 < 5$ KPa), HIF-1α protein levels are detectable through enhanced protein stability (Huang et al., 1996; Salceda and Caro, 1997; Pugh et al., 1997). Upon re-exposure to normoxia, HIF-α protein rapidly decays with a half-life of <5 min. By employing a panel of peptide

aldehydes that specifically inhibit the lysosomal proteases, calpains and the proteosome pathways, it has been shown that HIF-1α degradation occurs specifically via the ubiquitin-proteosome pathway (Huang et al., 1998). The target for the ubiquitination process is a regulatory element in the C-terminal region of HIF-1 termed the oxygen dependent domain (ODD). Only complete deletion of the ODD results in a stable HIF-1α expression. Partial deletions yield varying degrees of instability (Huang et al., 1998).

Until recently, it was not known how the ODD interacted with the ubiquitin-proteosome pathway. Maxwell et al., 1999 however showed that in renal carcinoma cell lines, the von Hippel-Lindau protein (pVHL) physically interacted with HIF-1α. This interaction occurs when hypoxia activates a prolyl hydroxylase enzyme which results in hydroxylation of a specific proline residue within the ODD (Ivan et al., 2001; Jaakkola et al., 2001). The bound pVHL is thought to mediate covalent attachment of multiple ubiquitin molecules to the protein complex and subsequent degradation by the 26S proteosome (Tanimoto et al., 2000).

In addition to direct protein interactions, phosphorylation appears to play a role in HIF-1α activation (Salceda et al., 1997a). The p42/p44 mitogen-activated protein kinases (MAPKs) (Richard et al., 1999) and the ERK MAPKs (Minet et al., 2000) have been shown to phosphorylate HIF-1α. This phosphorylation occurs primarily on serine residues, though phosphorylation has not been shown to be either sufficient or necessary for HIF-1α stabilisation (Sutter et al., 2000).

1.5.5. Mechanisms for hypoxic sensing

The nature of the oxygen sensor in multicellular organisms is still unknown. The studies of EPO regulation have fuelled the quest for a 'universal oxygen sensor'. Since the bioenergetic response to hypoxia is common to virtually all mammalian cells, a 'metabolic' sensor such as ATP depletion or acidosis might seem attractive. However since many of the adaptive responses in **Figure 1.5.1** occur at PO_2 levels well in excess of the P_{crit}, this is unlikely to be the case. At least 3 models for oxygen sensing have been proposed.

1.5.5.1. A haem-protein model for oxygen sensing

One postulated mechanism is ligand/receptor model, whereby oxygen binds to a specific receptor inducing downstream signalling. Given the peculiar properties of oxygen however, there are relatively few molecules to which it may bind. Oxygen is known to bind to and react with haem proteins and indeed in the bacterium *Rhizobium meliloti*, the oxygen sensor is an integral membrane haem-containing kinase (FixL) whose activity is modulated by O_2-binding (Gilles-Gonzales et al., 1991). Haem proteins have thus been suggested as candidate O_2-sensors in mammalian cells such that in hypoxic conditions the sensor switches from an'oxy' to a 'deoxy'-haemoprotein state (Goldberg et al., 1988).

The strongest evidence for this comes from studies in which carbon monoxide (CO) has been shown to disrupt the hypoxic signalling pathway (reviewed in Zhu and Bunn , 1999). CO displays avid binding affinity to ferrous haem groups in proteins such as haemoglobin, myoglobin and particular cytochromes. This binding appears to be remarkably specific to haem proteins. The finding that CO suppresses the expression of several hypoxia-mediated genes including EPO (Goldberg et al., 1988) and the expression of HIF-1 (Liu et al., 1998, Huang et al., 1999) is circumstantial evidence that the sensor is a haem protein. In addition, it is well established that certain transition metals including Co^{2+}, Ni^{2+} and Mn^{2+} can 'mimic' hypoxia, inducing expression of hypoxia-inducible genes via activation of HIF-1 (reviewed in Bunn and Poyton, 1996). If the oxygen sensor is a haem protein, then it is proposed that cobalt, nickel and manganese atoms, which do not bind oxygen, may substitute for the iron atom in the haem moiety and lock it in the 'deoxy' conformation.

There are however several lines of evidence that are not easily accommodated by this relatively simple model of direct oxygen sensing. Similarly to transition metals, the iron chelator desferrioxamine (DFO) 'mimics' hypoxia in a non-additive manner, suggesting a common pathway (Wang and Semenza, 1993A). Haem iron is however non-chelatable. Oxygen has been shown to directly interact with iron-sulphur clusters (Iwai et al., 1998), and there are examples of non-haem ferroprotein sensors in which labile iron atoms are

susceptible to chelation (Spiro and Guest, 1990). The initial report that HIF-1 contained non-haem iron would have clearly implicated this protein as a direct oxygen sensor (Srinivas et al., 1998); the authors however were unable to reproduce the data (Salceda and Caro, 1999). Nonetheless PAS domains are utilised in oxygen-sensing in certain prokaryotic organisms (reviewed by Taylor and Zhulin, 1999) which implies that HIF-1 activity may indeed be directly influenced by molecular oxygen concentration.

1.5.5.2. Cellular reduction-oxidation (redox) models for oxygen sensing.

Rather than direct oxygen sensing, an alternative model is the sensing of a metabolite of oxygen. In this model changes in oxygen concentration result in a shift in the redox state of the cell. Within this model there are two distinct mechanisms postulated.

The first proposes that the sensor may be a flavo-haem protein which functions as an NAD(P)H oxidase, chemically reducing oxygen to superoxide anion (O_2^-) which in turn is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Thus as oxygen concentration falls, so too does the production of O_2^- and H_2O_2, providing a redox signal for hypoxia. Fandrey et al. (1994) showed that in Hep3B cells, catalase increased expression and H_2O_2 reduced expression of EPO, suggesting a central regulatory role for reactive oxygen species (ROS) in oxygen sensing. A similar integral relationship between ROS and NF-κB activation has been proposed (reviewed by Li and Karin, 1999). Indeed as with NF-κB, HIF-1α contains a conserved redox-sensitive cysteine residue (Ema et al., 1999). The effect of DFO in this model may be reconciled by its effect on chelating free iron which catalyses the generation of ROS from H_2O_2 via the Fenton reaction. Hence DFO would reduce ROS generation and mimic hypoxia. Against this model is the knowledge that many antioxidants fail to mimic the effects of hypoxia on gene expression (Wenger, 2000), and indeed several antioxidants inhibit HIF-1 and hypoxic signalling (Haddad and Land, 2000; Chandel et al., 1997).

In the NAD(P)H model of sensing, a membrane bound NAD(P)H oxidase generates ROS in direct proportion to environmental oxygen. Hence, hypoxia would result in a net reduction in ROS generation. There is now a substantial body of evidence that this model

may be most relevant in Type 1 carotid body cells and pulmonary neuroepithelial bodies, sites of oxygen sensing in mammals (Gonzalez et al., 1994, Youngson et al., 1993). In the former, studies employing direct spectral analysis provided evidence that the oxygen sensor is a cytochrome *b*-like NAD(P)H oxidase (Acker, 1994) and patch clamp experiments localised it to the plasma membrane (Ganfornina and Lopez-Barneo, 1991) Diphenylene iodinium (DPI), an inhibitor of NAD(P)H oxidases (and other flavoproteins), stimulates depolarisation of carotid (Cross et al., 1990) and neuroepithelial (Youngson et al., 1993; Fu et al., 2000) bodies, as predicted by this model.

The role of an NAD(P)H oxidase as a *universal* oxygen sensor however is disputed. Patients with genetic subtypes of chronic granulomatous disease (CGD), who have absent or abnormal NAD(P)H subunits, exhibit normal oxygen sensing. Moreover elegant studies in knock-out mice and specific NAD(P)H subunit-deficient cell lines have demonstrated normal oxygen sensing (Wenger et al., 1996, Archer et al., 1999). In hypoxic hepatoma cell lines, DPI inhibits HIF-1 and downstream genes when the opposite effect would have been predicted by this model (Gleadle et al., 1995).

The alternative model of oxygen sensing via changes in redox state implicates the mitochondrial electron transport chain (ETC) complex IV (cytochrome c and cytochrome oxidase complex, also referred to as cyt aa_3) in a pivotal role. The mitochondrion is the primary site for oxygen metabolism and hence a role in oxygen sensing might seem logical. Under hypoxic conditions, the V_{MAX} of cyt aa_3 decreases and the K_m increases (Chandel et al., 1996), hence the reduction of oxygen to H_2O is inhibited. This 'blockade' of the ETC results in release of electrons upstream at complex III (b-c_1 complex). These electrons generate increased O_2^- via univalent transfer to oxygen. In hepatocytes and cardiac myocytes, graded decreases in PO_2 from 4.5 to 1 KPa resulted in a progressive *increase* in ROS as measured by fluorescent probe. This effect was mimicked by cobalt and correlated with HIF-1 induction and EPO gene expression (Chandel et al., 1998). In ρ^0 cells, which lack mitochondrial DNA and ETC activity, hypoxia fails to induce ROS production or the expression of HIF-1 and downstream genes (Chandel and Schumacker, 1999). This model of sensing conflicts directly with that described above in which a cytochrome *b*-like oxidase generates *decreased* levels of ROS during hypoxia.

Indeed recent studies also report normal HIF-1α expression in a range of mutant ρ^0 cells (Vaux et al., 2001; Srinivas et al., 2001). The conflict over the role of the mitochondrial chain in hypoxic sensing remains unresolved.

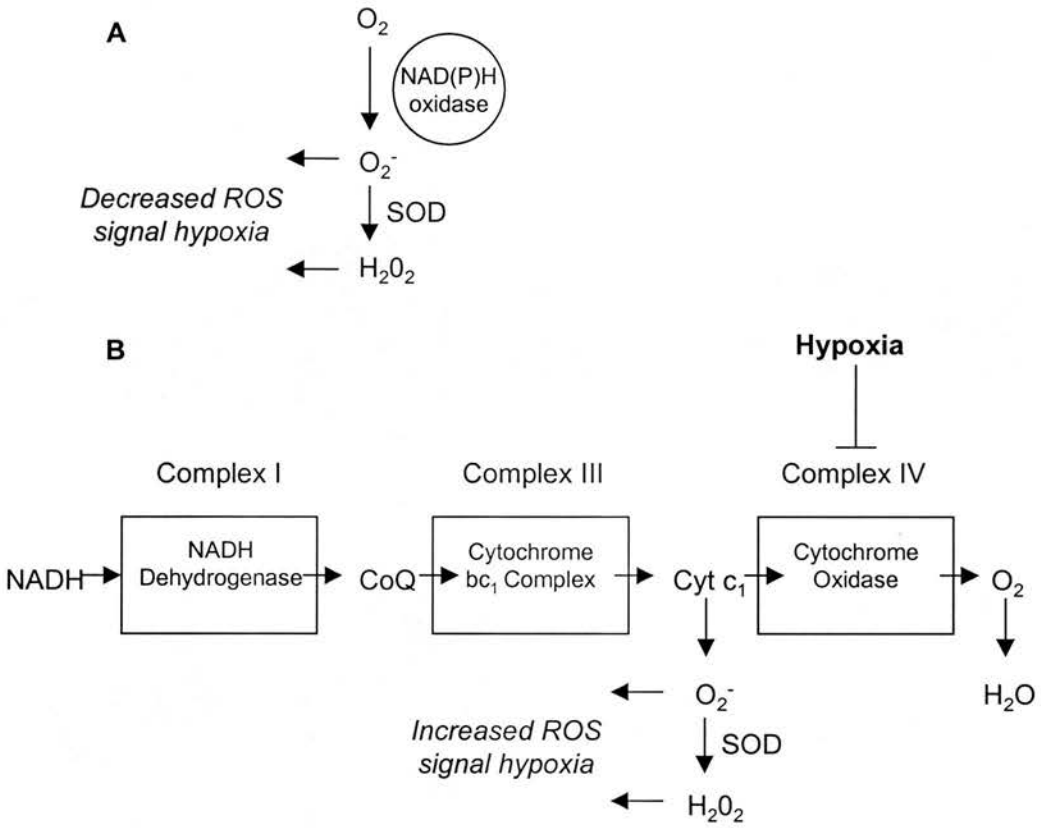
1.5.5.3. A nitric oxide (NO) model for oxygen sensing

Finally, in a novel approach utilising model organisms to explore the hypoxic sensing mechanism, Wingrove and O'Farrell (1999) have demonstrated that in *Drosophila melanoganster*, both acute and chronic hypoxic responses are impaired in flies in which the nitric oxide (NO) metabolism is disrupted either genetically or pharmacologically. For example flies exposed to the nitric oxide synthase (NOS) inhibitor L-NAME have impaired hypoxia-induced cell cycle arrest, but the hypoxic effect can be induced by exogenous expression of NOS.

The evidence for a direct role for NO in hypoxic sensing in mammals is largely circumstantial: the nitric oxide synthase gene is itself HIF-1 regulated (Melillo et al., 1995); NO inhibits HIF-1 induction (Sogawa et al., 1998, Huang et al., 1999) and prevents hypoxic upregulation of relevant genes (Ohigashi et al., 1993, Liu et al., 1998); NO has been shown to inhibit the activity of the mitochondrial ETC complex IV (Clementi et al., 1999); lastly NO has been shown to be produced endogenously at low levels by mitochondria (Giulivi, 1998), raising the notion that NO:oxygen ratio at mitochondrial level is a critical parameter in oxygen sensing. Only recently has there been data to support a direct coupling between oxygen sensing and NO signalling (Eu et al., 2000)

It is possible that rather than a single universal oxygen sensor, the response to hypoxia may depend upon the orchestrated response of several 'sensing mechanisms'. It is also apparent that the balance of sensing mechanisms may vary between cell phenotypes, for example between specialist oxygen sensing depolarisable cells and general non-depolarisable cells.

Figure 1.5.2



Legend for Figure 1.5.2. Redox models for oxygen sensing.

A. The oxygen sensor is a membrane-bound NAD(P)H oxidase that generates ROS in direct proportion to environmental oxygen. Thus, hypoxia results in reduced ROS and signalling.

B. The oxygen sensor resides within the mitochondrial electron transport chain, specifically within complex IV. Hypoxia inhibits the activity of cytochrome oxidase, blockading normal electron transport and generating increased ROS.

1.5.6. HIF-1 mediated gene expression

Following the identification of the HRE in the EPO gene, it became apparent that several other genes contain within their promoter or enhancer regions potential binding sites for HIF-1. Hypoxia regulated genes, in which a functional HIF-1 binding site has been definitively identified, or in which hypoxic induction can be mimicked by transition metals or DFO (thus implicating HIF-1 in transcriptional regulation), are summarised in **Table 1.5.6**. The majority of these studies have been in non-human cell lines, and it is apparent that hypoxic regulation of genes varies between cell lines (Gleadle and Ratcliffe,

1998; Lewis et al., 1999; Faller et al., 1999). The core sequence 5'-RCGTG-3', common to all the genes which have been shown to bind HIF-1, may be present in either the sense or antisense strand. However, for efficient hypoxic regulation, additional flanking regions around the HIF-1 binding site are necessary. For example the EPO, VEGF and ALDA genes all contain a site 5'-CACAG-3' close to the HIF-1 site which, if disrupted, prevents hypoxic upregulation. Hence it is likely that a functional HRE consists of at least a pair of contiguous transcription factor binding sites, at least one of which binds to HIF-1 (Semenza et al., 1996).

In the EPO gene the HRE is sited in an enhancer region 3' of the poly-A site. For most other genes studied the HRE is sited 5' of the transcription initiation site. The distance from this site however may be short, as in the endothelin-1 gene (-118 nt upstream) or relatively long, as in the transferrin gene (-3.5Kb upstream). Once bound to its concensus site, HIF-1 is able to recruit essential transcriptional coactivators such as the histone acetyltransferases CBP/p300 (Arany et al., 1996; Kallio et al., 1998; Ebert and Bunn, 1998) and transcription intermediary factor 2 (TIF2) and steroid receptor coactivator-1 (SRC-1) (Carrero et al., 2000).

Table 1.5.6.

Process	Gene Product	References
Erythropoiesis	Erythropoietin (EPO)	Firth et al., 1994, Wang and Semenza, 1993
Glycolysis	Aldolase A (ALDA)	Firth et al., 1994; Semenza et al., 1994; Ebert et al., 1995; Firth et al., 1995; Semenza et al., 1996; Iyer et al., 1998
	Enolase 1 (ENO1)	
	Lactate dehydrogenase A (LDHA)	
	Phosphofructokinase L (PFKL)	
	Phosphoglycerate kinase 1 (PGK1)	
	Pyruvate kinase M	
	Glyceraldehyde-3-phosphate dehydrogenase	Iyer et al., 1998; Graven et al., 1999
Glucose transporters	Glucose Transporter (GLUT) 1,2*,3	Ebert et al., 1995; Iyer et al., 1998
	α_{1B}-adrenergic receptor	Eckhart et al., 1997
	Adrenomedullin	Nguyen and Claycomb, 1999
Energy metabolism	Adenylate kinase 3	O'Rourke et al., 1996; Wood et al., 1998
Growth factors	Vascular endothelial growth factor (VEGF)	Goldberg and Schneider, 1994; Gleadle et al., 1995; Liu et al., 1995; Levy et al., 1995; Forsythe et al., 1996
	Flt-1 (VEGF receptor-1)	Gerber et al., 1997
	Platelet derived growth factor (PDGF) A and B	Kourembanas et al., 1990; Gleadle et al., 1995
	Placental growth factor (PLGF)*	Gleadle et al., 1995; Khaliq et al., 1999
	TGF-β3	Caniggia et al., 2000
Vasomotor regulation	Inducible nitric oxide synthase (iNOS)	Melillo et al., 1995; Melillo et al., 1997; Palmer et al., 1998
	Constitutive nitric oxide synthase (cNOS)*	Phelan and Faller 1996
	Endothelin-1†	Bodi et al., 1995; Hu et al., 1998
	Haemoxygenase-1 (HO-1)	Hernandez-Eyssen et al., 1996; Lee et al., 1997
Iron metabolism	Transferrin†	Rolfs et al., 1997
	Transferrin receptor gene	Lok and Ponka, 1999, Tacchini et al., 1999
Regulation of HIF-1 function	P35srj (CBP/p300 antagonist)	Bhattacharya et al., 1999
Cell cycle regulation	p21	Carmeliet et al., 1998
Others	Caeruloplasmin	Mukhopadhyay et al., 2000
	Insulin-like growth factor (IGF) binding protein -1	Tazuke et al., 1998; Feldser et al., 1999
	Peroxisome Proliferator-activated receptor α (PPAR)*	Narravula and Colgan, 2001

Legend for Table 1.5.6. Genes regulated by hypoxia in which HIF-1 has been implicated

* These genes are downregulated by hypoxia. A critical role for HIF-1 regulation in cNOS, GLUT-2 and PLGF downregulation is not established. PPAR is the only gene shown to be downregulated by a HIF-1 dependent mechanism

† One or more of the HIF-1 binding sitesare present on the antisense strand

Additional notes:

VEGF - mRNA transcription and stability is increased under hypoxic conditions
Flt-1 - may be regulated by HIF-2 rather than HIF-1.
HO - HIF-1 essential for regulation in rat vascular smooth muscle cells but not in CHO cells. AP-1 and other transcription factors are also relevant in hypoxic regulation of HO

Given the novel properties of HIF-1 and its clear involvement in the regulation of many genes critical to cell survival, HIF-1 has been termed a master regulator of hypoxic responses (Semenza et al., 1999). However, recent evidence suggests that HIF-1α expression is not exclusively hypoxia-dependent. As with many other transcription factors, HIF-1 α expression may also be induced by inflammatory mediators and growth factors (Haddad and Land, 2001; Richard et al., 2000; Zelzer et al., 1998). Conversely, there is growing evidence that transcription factors such as AP-1 and NF-κB, most commonly associated with cell survival and inflammatory gene expression, may be oxygen-regulated. The role of hypoxia in the regulation of AP-1, NF-κB and other transcription factors is discussed in **Chapter 5.3**.

1.6. HYPOTHESES AND AIMS OF THESIS

ARDS represents the severe end of a spectrum of lung injury that evolves over a period of hours or days in a subgroup of at-risk. In clinical studies of trauma patients in the very early at-risk period of ARDS, soon after the initiating insult, we have observed raised levels of intrapulmonary interleukin-8 (IL-8), but not other inflammatory cytokines, are associated with subsequent progression to ARDS ($n=56$, $P<0.001$). The high IL-8 levels were detected within a few hours (range 0.75 - 4 hr) of the trauma incident. Immunohistochemical analysis implicated the alveolar macrophage as a potent source of intrapulmonary IL-8. The mechanisms by which IL-8 may be rapidly generated in this clinical setting are unknown.

Our clinical observations suggest that events occurring in the immediate aftermath of a trauma incident contribute to the generation of IL-8 in macrophages. I hypothesised that clinically relevant physiological events may include:

1) A neuro-endocrine 'stress' response to major trauma. This would result in the rapid intrapulmonary and systemic release of clinically relevant stress mediators including catecholamines and neuropeptides that may stimulate the macrophage to generate IL-8.
2) Acute tissue hypoxia and hyperoxia. By the time of sampling, the trauma victims were likely to have undergone a period of sustained tissue hypoxia secondary to head-injury, atelectasis and lung contusion and subsequent resuscitation with delivery of high flow oxygen. I hypothesised that hypoxia / hyperoxia would act as a direct multiple-stimuli or 'hits' to generate IL-8 in macrophages.

To test these hypotheses perform the following studies:

1) I aimed to study the potential for a range of 'stress' mediators to stimulate IL-8 generation in human monocyte-derived macrophages. I aimed to develop a reproducible *in vitro* model for altering macrophage oxygenation to study the effects of acute hypoxia and hyperoxia on IL-8 generation.

2) I aimed to develop a novel animal model of direct and indirect acute lung injury in which to specifically test the effect of acute hypoxia / hyperoxia on intrapulmonary IL-8 generation.

3) I aimed to study the transcriptional regulation of the IL-8 gene in response to acute hypoxia in human macrophages

CHAPTER 2

MATERIAL AND METHODS

2.1. REAGENTS

The reagents used in these studies were purchased from the following sources:

Gibco Life Technologies (Paisley, Scotland, UK)

Iscove's Dulbecco's modified medium, without supplements, with L-glutamine (Iscove's MDM). Iscove's Dulbecco's modified Eagles medium, without supplements (Iscove's DMEM). Culture supplements penicillin (10000 IU/ml) / streptomycin (10 mg/ml), L-glutamine (200 mM), non-essential amino acids (100x), geneticin (G418 sulphate, 50 mg/ml), fetal calf serum. TRIzol® reagent. Acrylamide/bis (30% w/v). SDS (*Ultrapure*, 10%). Subcloning efficiency DH5α™ competent cells.

R&D Systems Europe Ltd (Oxon, UK)

Monoclonal (capture) mouse anti-human IL-8 (500 μg), TNF-α (500 μg) and IL-1β (500 μg) antibodies, dissolved in PBS and stored at –20°C. Biotinylated (detection) anti-human IL-8 (50 μg/ml), TNF-α (50 μg/ml) and IL-1β (50 μg/ml) antibodies, dissolved in PBS and stored at –20°C. Recombinant human TNF-α (10 μg) and IL-1β (5 μg) protein, reconstituted in PBS and stored at -70°C .

Promega (Southampton, UK)

RT-PCR reagents: AMV reverse transcriptase (5-10 U/μl), oligo-(dT) primer (0.5 μg/μl), rRNasin (40 U/μl), MgCl (25 mM), dNTPs (10 mM), T*fl* DNA polymerase (5 U/μl). Restriction enymes: *Pst* 1, *Bam*H 1, *Xba* 1, *Eco*R 1 with buffers. EMSA reagents: T4 polynucleotide kinase with 10x buffer, NF-κB consensus oligonucleotides. Transfection reagents: Tfx™-10 reagent, pSV-β-Galctosidase control vector, pCAT®-Control vector. Acryl-a-Mix® 6 polyacryamide gel solution. pGEM®-T vector.

Amersham Pharmacia Biotech (Bucks., UK)

Radio-labelling reagents: ReadyToGo™ DNA labelling beads (-dCTP), [α-^{32}P]-dCTP, [γ–^{32}P]-dATP, [α-^{32}P] UTP. Percoll (100%).

Boehringer Mannheim (E.Sussex, UK)

CAT and β-GAL ELISA systems. Complete™ protease inhibitor cocktail.

Other sources

Diff-Quick stain (Oberkochen, Germany). Streptavidin-peroxidase conjugate for use in ELISAs was from Dako (Cambs., UK). BCA protein assay kit for measurement of total protein was from Pierce (Rockford, IL, USA). Specific oligonucleotide sequences were purchased from MWG Biotech (UK) Ltd. DNA plasmid maxiprep (Quantum™) kit was purchased from BioRad Labs (Herts., UK). Nonidet P40 was purchased from BDH (Poole, UK). Monoclonal antibodies to p50, p65, c-Jun and c-Fos for use in EMSAs were purchased from Santa Cruz Biotech (CA., USA). Rabbit serum was obtained from SAPU (Lanarks., UK). RiboQuant® multi-probe RNase protection assay system (Pharmingen, San Diego, CA) was purchased through Becton Dickinson Ltd (Oxford, UK). Nylon membrane (Genescreen Plus) for transfer of RNA was obtained from Du Pont NEN (Brussels, Belguim). Rabbit capture and detection antibodies, and recombinant-rabbit IL-8 protein were a kind gift from Dr Caroline Hebert, Genentech Inc., CA., USA. HIF-1 expressing and non-expressing CHO-K1 cells were a kind gift from Prof Peter Ratcliffe, Oxford, UK. For the animal model of lung injury, Hypnorm® was obtained from Janssen-Cilag Ltd., High Wycombe, UK, midazolam (Hypnovel®) from Roche Products Ltd., Welwyn Garden City, UK and Pavulon® from Organon Labs. Ltd., Cambridge

All other reagents were purchased from Sigma Chemical Company (Poole, Dorset, UK).

The source of equipment used is cited within the text of the Methods (Chapter 2.2)

2.2. METHODS USED IN *IN VITRO* STUDIES

2.2.1 Cell Culture

2.2.1.1 Purification of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were purified according to the method of Haslett et al., (1985) with slight modification. Freshly drawn venous blood was collected into 50 ml polypropylene tubes primed with anticoagulant (4 ml 3.8% sodium citrate / 36 ml blood). Tubes were centrifuged (300g, 20 min) and the plasma carefully aspirated and used to prepare autologous serum by adding $CaCl_2$ (final concentration 20 μM) to plasma in a glass tube and incubating at 37°C for 1 hr. To the residual volume containing cell pellet, 5 ml of 6% dextran (MW 500,000, 37°C) and ~ 20 ml 0.9% saline (37°C) was added to a final volume of 50 ml. Tubes were mixed by gentle inversion and allowed to stand for 30 min resulting in red cells sedimentation. The leukocyte-rich upper layer was carefully aspirated and centrifuged (300g, 5 min), leaving a white-cell pellet. Isotonic Percoll was prepared by adding 1 part 10X PBS (-) to 9 parts neat Percoll. This isotonic solution was then diluted with PBS (-) to 81%, 70% and 55% solutions. The 70% solution (3 ml) was gently layered onto 3 ml of 80% solution in a 15 ml polypropylene tube. The white cell pellet was resuspended in 55% Percoll, gently layered onto the 70% solution, and the tubes centrifuged (700g, 20 min). PBMC were aspirated from the 55% / 70% Percoll interface and were washed twice in PBS (-) before counting by haemocytometer. Neutrophils sedimented at the 70% / 81% interface and red cells pelleted to the bottom of the tube.

The phenotype of the harvested cells was checked by flow cytometry with appropriate gating. Neutrophil contamination of PBMC was routinely <1%. Depending upon donor variability, monocytes constituted between 15 and 30% of the PBMC population.

2.2.1.2. Monocyte selection and culture to macrophages

PBMC were suspended in serum-free Iscove's modified Dulbecco's medium (MDM) supplemented with 50 U/ml penicillin and 50 mg/ml streptomycin at 4 x 10^6 / ml and 3 ml aliquots added to each well of 6-well plates. The plates were incubated at 37°C / 1 hr during which monocytes selectively adhered to the plastic. Non-adherent lymphocytes were then removed by washing with warm medium. Adherent monocytes were then overlayed with medium containing 10% autologous serum. For maturation to macrophages, cells were incubated for 5-7 days (37°C in humidified, 5% CO_2 atmosphere). The medium was replenished on day 3.

2.2.1.3. THP-1 cell culture

THP-1 human monocyte cells (a kind gift from Dr Simon Hart, University of Edinburgh) were maintained in suspension in Iscove's MDM containing 10% heat-inactivated FCS and supplemented with 50 U/ml penicillin and 50 mg/ml streptomycin. In experiments, 2 ml aliquots of cell suspension at a density of 1 X 10^6 cell per ml were plated per well of a 6-well plate.

2.2.1.4. Rabbit alveolar macrophage cell culture

Bronchoalveolar lavage (BAL) of rabbit lungs is described in **Chapter 2.3.5**. BAL fluid was centrifuged (400g, 6 min, 4°C) and the cell pellet washed once withcold PBS(-). In some cases a hypotonic red-cell lysis procedure was performed as follows; the cell pellet was suspended in sterile water (19 ml, 4°C) for exactly 20 seconds. Sterile 3.6% saline (6 ml, 4°C) was then rapidly added and mixed by inversion followed by centrifugation (6 min, 4°C). The phenotype of the lavaged cells was assessed by pelleting ~0.15x10^6 cells onto glass slides (Cytospin 2, Shandon Scientific, Cheshire, UK) and staining with Diff-Quick (these should be in the reagents section), a modified Wright-Giemsa stain. BAL fluid from normal rabbit lungs contained >98% alveolar macrophages (**Figure 2.2.1.4**). The cells were finally suspended at 0.5x10^6 cells/ml in Iscove's MDM supplemented with 50 U/ml

penicillin, 50 mg/ml streptomycin and 10% heat-inactivated FCS and 2 ml aliquots ($1x10^6$ cells) dispensed into 6-well plates. Alveolar macrophages were left in culture (37°C, 5% CO_2) for 48 hours to allow adherence to plastic prior to experimental manipulation.

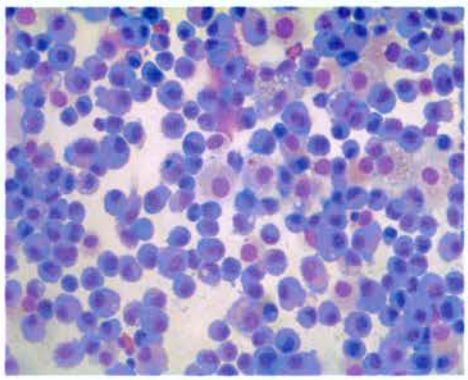


Figure 2.2.1.4. Cells recovered from bronchoalveolar lavage of healthy rabbit lungs.
Alveolar macrophages comprise ≥ 98 % of BAL cells. Cytospin stained with Diff-Quick (a modified Wright-Giemsa stain)

2.2.1.5. C4.5 and Ka13 CHO cell culture

C4.5 and Ka13 cells were a kind gift from Morwenna Wood (Oxford). These cells are derived from CHO K1 cells as described by Wood et al., (1998). Cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat -inactivated foetal calf serum (FCS), L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 mg/ml), non-essential amino acids (1x) and geneticin (G418, 500 µg/ml). Cells were grown in 162 cm^2 sterile flasks and serially passaged when 95-100% confluent.

2.2.1.6. Normoxic cell culture

In all experiments, 'normoxic' cell culture refers to culture in a standard humidified incubator at 37°C, 5% CO_2, 21% O_2.

2.2.1.7. Hypoxic cell culture

Two different incubators were available for hypoxic culture. The NAPCO hypoxic incubator (37°C, 0.1% O_2, 5% CO_2)(Precision Scientific Ltd., UK) was a standard

incubator in which the hypoxic environment was achieved through constant flushing with N_2. The MK3 anaerobic unit (37°C, 80% N_2 / 10% H_2 / 10% CO_2) (Don Whitley Scientific Ltd. UK) was a sealed chamber constantly flushed with gas mixture. This latter incubator maintains a near-anaerobic atmosphere (<25 ppm oxygen) through the reduction of oxygen by hydrogen in the presence of a palladium catalyst. A sealed interface allowed cultured cells to be accessed directly in the MK3 unit, without exposure to the environment. In contrast, transferring or manipulating cells in the NAPCO incubator could only be achieved by opening the chamber door and thus allowing air to enter the incubator. Brief periods of reoxygenation were therefore likely to occur in this system. Both incubators were used in preliminary experiments to asses the effect of hypoxic incubation on oxygen content and pH of culture medium (**Chapter 3**). However, unless otherwise stated, all data presented in this thesis pertaining to hypoxic incubation was derived from experiments using the MK3 anaerobic unit.

2.2.1.8. Hyperoxic cell culture

Hyperoxic incubation was achieved using portable air-tight 2 litre chambers which were flushed with 90% O_2 / 10% CO_2 at a rate of 4 L/min for 2 min and then sealed manually. The chambers were humidified by placing saturated paper towels in the chamber base. The sealed units were then placed in a 37°C incubator.

2.2.2. Measuring dissolved oxygen in medium

An OXEL-1 probe, which is based upon the electrode described by Clark and ISO_2 meter (World Precision Instruments, Inc. Florida, USA) was used to measure dissolved oxygen. Briefly, the OXEL-probe houses a platinum electrode and a silver counter / reference electrode inside a stainless steal sleeve. A gas permeable membrane fits over the sleeve and allows oxygen to diffuse through it, whereupon it is reduced at the platinum electrode. The magnitude of the electrical current generated is determined by the rate of diffusion to the electrode, which is proportional to the partial pressure of oxygen outside the membrane. Liquids, ions

and particulate matter are unable to pass through the membrane. The probe tip is 2 mm in diameter, and as such was not suitable for measuring PO_2 in the immediate peri-cellular vicinity. Instead, the tip was positioned ~5 mm above the cell surface.

The oxygen probe was calibrated by placing the tip in PBS at 37°C and bubbling either N_2 (0% oxygen) or room air (20.4% oxygen) at a rate of 2 L/min for up to 30 min to achieve equilibration. Measurement of PO_2 in samples was made by placing the 2-mm probe-tip 5 mm above the bottom of the plate.

In some instances, the results obtained with the OXEL-1 probe were compared to those derived using an automated gas analyser (model ABL-330, Radiometer, Copenhagen). This analyser simultaneously measured the partial pressure of O_2 and CO_2 and the pH in 250µl aliquot of sample fluid. The oxygen-sensing component of the automated analyser, as with the OXEL-1 probe, is based upon the Clark electrode.

2.2.3. Measurement of Lactate Dehydrogenase (LDH)

LDH assay, together with Trypan-blue exclusion, was used to measure cell viability. For each sample 2ml of Phosphate Buffer (PB), 0.1 mol/l., at pH 7.5 was placed in a 5ml Falcon tube and kept on crushed ice. The technique is based upon an assay originally described by Wrobleski and Landue (1955, LDH activity in blood PSEBM 90 210-213).

NADH (preweighed and damp protected, Sigma) was gently taken up in PB to a final concentration of 0.4 mg/ml; then 200 µl added to each Falcon tube. Samples of medium were centrifuged at 10,000 g for 3 min. A 700 µl aliquot was added to the Falcon and mixed well by inversion. Samples of cell lysates were obtained by scraping cells into 700 µl PBS containing 0.1% Nonidet-P and keeping the sample on ice for 30 min. The mixture was centrifuged at 10,000g for 3 min and the supernatant added to the Falcon.

The contents of the Falcon were transferred into a 3 ml cuvette and allowed to stand at room temperature for 20 minutes. The cuvette was then installed in the holder (at 37°C) of a Philips PU8 700 spectrophotometer and allowed 3 minutes to warm up before 100 µl of sodium pyruvate (22.7 mmol/l in PB) was added directly to the cuvette and mixed with a plastic paddle. The spectrophotometer was programmed to measure the rate of change of absorbance for light at 340 nm, determined and averaged over 3 minutes, sampling every 30 seconds. A standard curve was established using human erythrocyte LDH (Sigma), in the range 0.025 units to 0.5 units.

LDH assays were performed on fresh samples, or samples stored for a maximum of 24 hours at 4°C.

2.2.4 Enzyme-linked immunosorbent assay for human IL-8, TNF-α and IL-1β

Monoclonal and biotinylated anti-human IL-8, TNF-α and IL-1β antibodies were obtained from R&D Systems and the assays performed as per the manufacturer's protocol. Flat-bottomed 96-well microtitre plates were coated with 100 µl per well of monoclonal antibody (4 µg/ml for IL-8, TNF-α and IL-1β) in PBS (pH 7.4) overnight at room temperature. Wells were washed three times with wash buffer (0.05% Tween 20 in PBS) and 200 µl of 1% BSA in PBS added per well to block non-specific binding sites. Plates were incubated for 1 hour with blocking solution and wells subsequently washed three times. Samples were diluted if required in diluent buffer; 0.1% BSA, 0.05% Tween 20 in Tris buffered saline (20 mM Trizma base, 150 mM NaCl, pH 7.3). Samples aliquots (100 µl) added to wells in triplicate. Recombinant human (rh) TNF-α and IL-1β, obtained from R&D, were used to make up ten standard 1 in 2 serial dilutions starting at 10 ng/ml for TNF-α and 1 ng/ml for IL-1β. In the case of IL-8, rhIL-8 was obtained from NIBSC and serial dilutions started at 20 ng/ml. Standards were measured in duplicate. Control (blank) wells consisted of diluent buffer only. Plates were incubated at RT for 2 hours without shaking. After further washing, 100 µl per well of secondary biotinylated antibody (20 ng/ml for IL-8, 200 ng/ml for TNF-α and 100 ng/ml for IL-1β) was added and

the plates incubated for 2 hours. Plates were washed and incubated for 20 min with streptavidin-peroxidase conjugate (Dako, Denmark) diluted 1 in 20,000. After washing, substrate solution consisting of TMB 100 μg/ml, 4.5 mM H_2O_2 in 0.1 M sodium acetate-citrate pH 4.9 was added and the plates incubated in darkness to the desired extinction. The reaction was stopped with 50 μl of 0.5 M H_2S0_4. Plates were read at 450 nm (Dynatech MR5000).

2.2.5. Northern blotting

2.2.5.1. Preparation of diethyl pyrocarbonate (DEPC) water and RNase free equipment

DEPC water was prepared by adding 50 μl of diethyl pyrocarbonate in 500 μl ethanol to 500 ml MilliQ water and warming to 37°C with gentle stirring overnight in a fume hood. The water was then autoclaved (15 mins / 121°C / 200 KPa).
Pipette-tips and glass-ware were rendered RNase-free by autoclaving. Non-autoclavable items were treated with 3% H_2O_2 overnight, or RNaseZAP™. Solutions were constituted in DEPC-treated water.

2.2.5.2. Extracting RNA from cultured cells

RNA from cultured cells was extracted using Trizol™, as per the manufacturers protocol. This method is a based on the phenol / chloroform extraction technique described by Chomczynski and Sacchi, 1987. Adherent cells were lysed directly in 6-well plates by addition of 800 μl of Trizol™ reagent per well for 5 min at RT. The lysate was transferred to 1 ml tubes and 200 μl chloroform added and vortexed. The mixture was incubated at RT for 3 min then centrifuged at 12,000g for 15 min at 4°C. The resulting upper RNA-containing aqueous phase was gently aspirated, without disturbing the interphase (DNA and lipid-soluble proteins) or lower organic phase (protein).

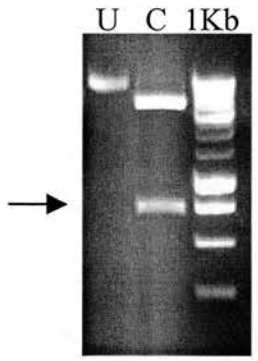
The RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol and incubation for 10 min at RT (or overnight at -20°C) followed by centrifugation at 12,000g for 10 min at 4°C. The resulting RNA pellet was washed in 75% ethanol, centrifuge at 7500g for 5' at 4°C and allowed to air dry. RNA was dissolved in DEPC water and stored at -70°C. The quantity of RNA was measured by spectrophotometry (Pharmacia Biotech, Ultraspec 2000). RNA purity was determined by measuring $A_{260/280}$ (typically 1.6-1.8 using this method) and by resolving 1 μg of RNA in a non-denaturing 1.5% agarose gel and observing resolved 11s and 18s bands.

2.2.5.3. Constructing a human IL-8 cDNA probe for northern blotting

A human IL-8 cDNA probe had previously been cloned into a pBS+ vector (Bluescribe, Stratagene, CA) by Dr Mark Lawson, Rayne Laboratory, University of Edinburgh. Briefly, total RNA from human peripheral blood monocytes stimulated with LPS was extracted and RT-PCR performed using the primers -ATT T**CT GCA G**CT CTG TGT GA- (downstream, sense) and -TGT **GGA TCC** TGG CTA GCA GA- (upstream, antisense). These primers contained internal restriction sites for Pst-1 and Bam H1 respectively (in bold), and spanned the 4 exons and three introns of the IL-8 gene (Schmid and Weissmann, 1987).

A 747 bp product was cloned into a pBS+ plamid vector (Bluescribe, Stratagene, La Jolla, CA) precut with Pst1 and BamH1. The successful clone, JB-1, was transformed in bacteria, cultured in LB medium and pure plasmid DNA derived using the Promega DNA Maxiprep™. JB-1 plasmid (2 μg) was incubated with 40U BamH1, 40U of Pst-1 in 1x BamH1 buffer for 37°C for 2 hours. The DNA was then run on an agarose gel (**Figure 2.2.5.3**). The 747 bp IL-8 cDNA band was cut from the gel under UV light, and purified using the Promega Wizard™ PCR Prep system.

Figure 2.2.5.3



Legend for Figure 2.2.5.3 IL-8 cDNA probe (747 bp) cut from JB-1 plasmid. Plasmid was cut as described in text, to yield a 747 bp human IL-8 cDNA product for use in northern blotting. (U=uncut plasmid, C= cut plasmid)

2.2.5.4. Resolving RNA samples in formaldehyde gel

RNA samples (15 μg in ≤ 15 μl) were prepared by adding 15 μl of RNA loading buffer with ethidium bromide, heat-denaturing the sample/buffer mixture at 65°C for 15 min and immediately plunging in ice. Samples were loaded onto a 1.2% 2x MOPS / formaldehyde–agarose gel (40 mM 3-morpholinopropanesulphonic acid, pH7.0, 10 mM sodium acetate, 1 mM EDTA, 2 M formaldehyde) and electrophoresed at 100V for 2 hours in 1x MOPS. The gel was then viewed under UV light to assess consistency in RNA loading between samples.

2.2.5.5. Capillary transfer of RNA to membrane

Following electrophoresis, the formaldehyde gel was immersed in 0.05 M NaOH for 10 min with gentle agitation. The gel was than gently washed twice in DEPC water for 10 min and finally in 2x SSC for 20 min. This process aided cleavage of larger (>800 bp) species of RNA and removed formaldehyde from the gel. The nylon membrane (Du Pont NEN Genescreen Plus) was prepared by soaking in DEPC water for 10 min followed by immersion in 2x SSC for at least 20 min. The blotting apparatus was constructed as per **Figure2.2.5.5.** Following overnight transfer, the membrane was placed in 2x SSC, enclosed in Saran-Wrap™ and wet cross-linked under UV-light (600μW/cm^2 at 254nm for 5 min).

Figure 2.2.5.5

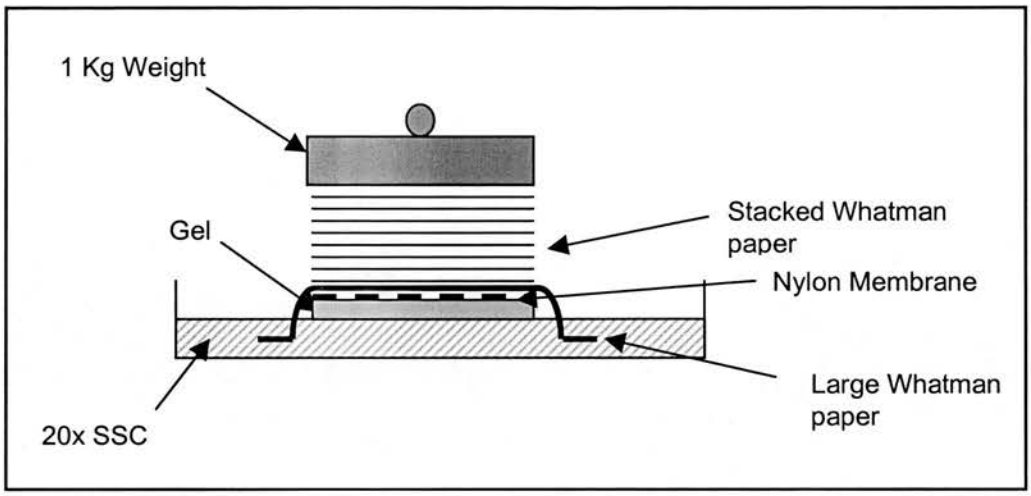


Figure 2.2.5.5. Apparatus for northern blotting

2.2.5.6. Radio-labelling cDNA probe

The cDNA probe was labelled using 'Ready-to-go' DNA labelling beads (Pharmacia Biotech) as per the manufacturers protocol. Twenty nanograms of DNA denatured by heating to 98°C for 5 min then plunged into ice. The linearised DNA was then added to reconstituted labelling beads and incubate at 37°C for 30 min to 1 hr with 5 µl [α-^{32}P]dCTP (3000Ci/mmol).

2.2.5.7. Membrane hybridisation

The cross-linked membrane was pre-hybridised for 2 hr at 65°C in 20 ml 1x Chuch and Gilbert buffer [0.5 M $NaHPO_4$, 7% SDS and 1 mM EDTA (pH 7.2)]. Fresh buffer containing [α-^{32}P]dCTP-labelled cDNA probe was then added and hybridisation continued for 18 hours at 65°C. The membranes were washed three times in 0.1x SSC, 0.1% SDS, once at 65°C and twice at room temperature. Membranes were exposed to Biomax MR film (Kodak) with intensifying screens at –70°C. Membranes were subsequently stripped with boiling 0.1% SDS and re-probed with a housekeeping 18s cDNA probe.

2.2.6. Multi-probe RNase Protection Assay (RPA)

The detection of multiple mRNA species expression in total RNA was achieved using multiple RNA riboprobe templates and the RiboQuant assay system (Pharmingen, San Diego, USA). The assay was performed as per the manufacturers protocol.

2.2.6.1. Multi-probe synthesis

All reagents were bought to room temperature unless otherwise stated. The following reagents were co-incubated at 37°C for 1 hour: 1 µl RNasin, 1 µl GACU oligonucleotide pool, 2 µl DTT, 4 µl 5X transcription buffer, 1 µl RPA template set, 10 µl [α- ^{32}P]UTP and 1 µl T7 bacteriophage RNA polymerase. This reaction generates highly specific radiolabelled antisense RNA in large quantity from specific cDNA templates. The reaction was terminated by adding 2 µl of DNase, and the mix incubated for a further 30 min at 37°C. Protein was removed from the reaction mix using phenol/chloroform: 26 µl 20 mM EDTA, 25 µl Tris-saturated phenol, 25 µl chloroform:isoamyl alcohol (50:1) and 2 µl yeast tRNA was added to the tube, vortexed into an emulsion and centifuged at 12,000g for 5 min at RT. The upper, riboprobe-containing, aqueous phase was removed and the lower phase discarded. A further 50 µl chloroform:isoamyl alcohol (50:1) was added to the riboprobes, the mixture vortexed and centrifuged at 12,000g for 2 min at RT. The upper phase was removed and unincorporated radiolabel removed by precipitation in 250 µl ice-cold 100% ethanol in the presence of 50 µl 4 M ammonium acetate, the sample centrifuged at 12,000g for 15 min at 4°C. The waste supernatant was removed, 100 µl ice-cold 90% ethanol added to the pellet and the tube spun at 12,000g for 5 min at 4°C. After removal of the supernatant, the pellet was air-dried for 10 min and dissolved in 50,µl hybridisation buffer. The labelling efficiency was determined by measuring the cpm/µl in a 1 µl sample in a scintillation counter. An acceptable lower limit was ~ 3 X 10^{-5} Cherenkov counts/µl.

2.2.6.2. Hybridisation of riboprobes with RNA and RNase treatment.

The purified riboprobes were allowed to hybridise in excess with sample total RNA (2 µg per reaction) to generate probe annealed to homologous sequences in the sample RNA. Hybridisation was performed at 56°C overnight. Remaining non-hybridised single stranded RNA was digested with an RNase specific for single-stranded species (RNase A in RNase A buffer, 45 min at 37°C). The double-stranded RNA / RNA hybrids were recovered by ethanol precipitation as above. Prior to loading onto the gel, 5 µl of 1X loading buffer was added to each sample and the mixture heated to 90°C for 3 min and immediately plunged into ice.

2.2.6.3.Gel resolution of protected species

The RNA / RNA hybrids were resolved on a denaturing polyacrylamide gel. Glass plates were cleaned with 70% ethanol and the short plate siliconized. The denaturing gel-mix was made up as follows to a final concentration of 5% acrylamide:

- 8.85 ml 40% acrylamide
- 9.31 ml of 2% bis acrylamide
- 7.45 ml of 10X TBE
- 35.82 g of Urea
- Made up to 74.5 ml with distilled water

Immediately prior to pouring, 450 µl of ammonium sulphate (10%) and 60 µl of TEMED was added to the gel-mix. Following polymerisation, the gel was placed in a vertical electrophoresis apparatus (Biorad-Quentum) and prerun at 40 watts constant power for 45 min in 0.5X TBE running buffer. The desired gel temperature was ~50°C. The samples were loaded onto the gel, as was a dilution of the template probe set (~ 1000 cpm / lane) in loading buffer which would serve as ladder. The gel was run at 50 watts constant power until the leading edge of the bromophenol blue reached 30 cm. After adsorbing onto to Whatman paper and covering in Saran wrap,

the gel was dried under vacuum for 1 hour at 80°C. Dried gels were exposed to X-ray film and densitometry performed.

2.2.7. Electromobility shift assay

Total nuclear proteins were extracted from cells using a method modified from that previously described (Andrews et al, 1991). Macrophages were kept on ice and scraped into cold buffer containing 10 mM HEPES – KOH pH 7.8, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA protease inhibitor cocktail (Complete™ , Boehringer Mannheim). The cells were allowed to swell on ice for 15 min then lysed by adding 0.4% Nonidet P40 and centrifuged (12,000 *g* for 1 min at 4°C). The supernatant was carefully removed and the pellet resuspended in 50 µl of cold buffer containing 50 mM HEPES – KOH pH 7.8, 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA and protease inhibitor cocktail. Following 20 min incubation at 4°C with gentle agitation and centrifugation (12,000 *g* for 1 min at 4°C) the supernatant was carefully removed and protein content assayed by BCA protein assay (Pierce, Rockford, IL). The binding reaction was performed at RT as follows: 4 µg aliquots of protein were incubated for 5 min in binding buffer containing 5 mM Tris, 0.5mM EDTA, 8% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT and 1.2 µg of polydI-dC and then a further 15 min with an appropriate [$\gamma-^{32}$P]-labelled oligonucleotide. In the case of supershift assays nuclear extracts were first co-incubated at 4°C with rabbit anti-human polyclonal IgG antibodies to p50 and p65 (Santa Cruz) for 4 hours or with antibodies to c-jun and c-fos (Santa Cruz) for 20 hours in binding buffer minus DTT. These were subsequently incubated at RT with appropriate [$\gamma-^{32}$P]-labelled oligonucleotide in the presence of 0.5 mM DTT. The reaction mixtures were separated on a 6% polyacrylamide gel at 180 V at room temperature in 0.5% TBE buffer. After drying, gels were exposed to X-ray film and densitometry performed. The oligonucleotide probes used are shown in **Table 2.2.7**

Table 2.2.7

Name	Sequence (sense strand only shown)
C/EBP (IL-8 gene) Wild-type Mutant	 5'-CAT**CAGTTGCAAATCG**TTAAC-3' 5'-CAT**CAGTagCcAAtcTcG**AAC-3'
AP-1 (IL-8 gene) Wild type Mutant	 5'- AGTGTGA**TGACTCA**GGTTTGC-3' 5'-AGTGTGA**TatCTgt**GGTTTGC-3'
NF-κB (IL-8 gene) Wild type Mutant	 5'- TCGT**GGAATTTCCT**CTGACAT-3' 5'-TCGT**taAcTTaCCTCT**GACAT-3'
AP-1 consensus	5'- CGCTTGA**TGAGTCA**GCCGGAA- 3'
NF-κB consensus	5'- AGTTGA**GGGGACTTTCC**CAGGC-3'

Table 2.2.7 Oligonucleotide probes used in EMSA studies

2.2.8. Extraction of total cellular protein

Total cellular protein was extracted from cells using the Trizol™ reagent, as used for RNA extraction. This method offered the advantage of simultaneously obtaining total cellular protein and RNA from a single sample of cells. The procedure is as described for RNA extraction (**2.2.5.2**). Following chloroform phase separation of the RNA, 0.3 ml 100% ethanol was added per 1 ml of Trizol initially used was added to the DNA- and protein-containing phases. Samples were stored for 2-3 min at RT and the DNA precipitated by centrifuge at 2000g (5000 rpm) for 5' at 4°C. Protein in the supernatant was precipitated in isopropanol (10 min incubation at RT followed by centrifugation 12,000g/10 min/4°C. The protein pellet was washed 3-times in 0.3 M guanidine hydrochloride in 95% ethanol, with a final wash in 100% ethanol. After air-drying, the pellet was dissolved in 1% SDS.

2.2.9. Measurement of protein concentration

Cellular protein extracts were quantified using a BCA protein assay (Pierce, IL, USA). This assay is based on the ability of protein in the test samples to cause a reduction of Cu^{2+} to Cu^{+} and bicinichoninic acid (BCA) to chelate Cu^{+} forming a purple compound which may be measured spectrophotometrically at 526 nm. Samples were diluted up to 1 in 10 in dH_2O and 10µl incubated with 200 µl of test reagent for 30 min at 37°C. Plates were analysed by automated plate reader (MR5000, Dynatech, UK). Samples were assayed protein concentration measured with reference to standard curves derived from BSA standards.

2.2.10.Western blotting for HIF-1α

This method was provided by Dr M. Wiesener (Wellcome Trust Centre for Human Genetics, Oxford, UK), who also kindly performed preliminary blotting experiments. Total protein extracts (80 µg) were resolved in an SDS/10% polyacrylamide gel and transferred to Immobolin P (Millipore, Bedford, MA) overnight in 10 mM Tris, 100 mM glycine, 10% methanol and 0.05% SDS. Membranes were blocked with PBS containing 5% fat-free dried milk and 0.1% Tween 20. Immunoblotting for HIF-1α was with MoAb 28B (4 µg/ml)(Wiesener et al., 1999). Detection was with HRP-conjugated goat anti-mouse Igs and enhanced chemiluminescence (SuperSignal Ultra; Pierce, Rockford IL). After analysis, membranes were stained with Ponceau S to verify equal protein loading and transfer.

2.2.11. Immunofluorescent staining for E-selectin and CD2

This was performed on C4.5 cells, the phenotype of which is described in **Chapter 6**. Cells cultured in 6-well plates were washed twice with cold PBS (-) and 1 ml PBS (-) / EDTA 0.002 M / BSA 0.1% added to each well. The plates were incubated on ice for 30 min. Cells were aspirated, transferred to a 1 ml Eppendorf tube and centrifuged at 10,000 g for 5 min at 4°C. The cell pellet was resuspended in flow

buffer (0.1% BSA / 0.1% sodium azide) at a concentration of $1X10^6$ / ml. Aliquots of 100,000 cells were added to flat-bottomed 96-well plates that were subsequently centrifuged at 200 g for 2 min. The supernatant was removed by flipping the plates, and cells incubated with monoclonal antibodies (50 µl) to human E-selectin (1 in 100), human CD2 (neat) and appropriate controls (anti-human CD5 (SAPU) and anti-mouse MOPC) for 30 min on ice. Washing was repeated twice with flow buffer as above, and the secondary antibody, goat anti-mouse Ig (F(ab')2 (1 in 40 in flow buffer) (DAKO). Following a further 30 min incubation on ice and two washes, 200 µl flow buffer was added to each well and the resuspended cells analysed by FACS.

2.2.12. Transfection experiments with the human CAT-linked IL-8 promoter

The human IL-8 wild type promoter (280 bp), cloned into the pCAT basic vector, was obtained (a kind gift from Prof. R Strieter, CA). The pCAT basic vector map, and the control vectors, pCAT®-Control and pSV-β-Galctosidase control are shown in **Figure 2.2.13.**

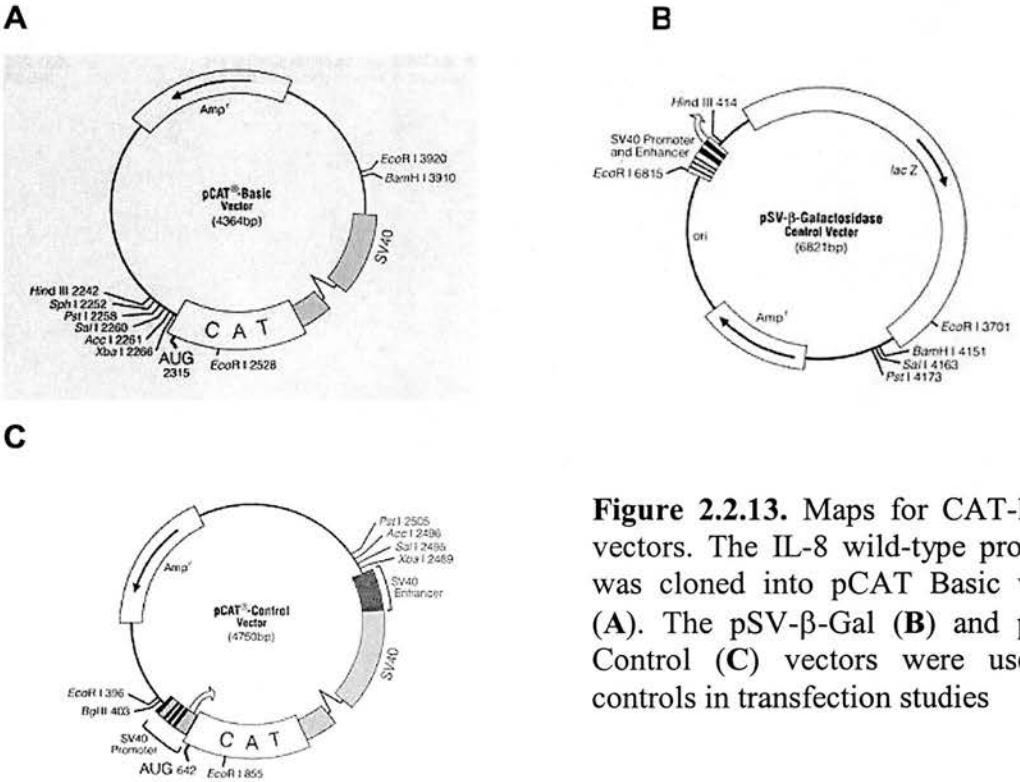


Figure 2.2.13. Maps for CAT-linked vectors. The IL-8 wild-type promoter was cloned into pCAT Basic vector (**A**). The pSV-β-Gal (**B**) and pCAT Control (**C**) vectors were used as controls in transfection studies

2.2.12.1. Transformation of the IL-8 CAT-linked promoter in bacteria

A 50 µl aliquot of DH5α (E.Coli) competent cells was thawed on ice and 10 µg of vector DNA added and gently mixed by pipetting. The cells were incubated on ice for 30 min followed by heat-shock for 20 sec at 40°C. Nine hundred and fifty µl of room temperature S.O.C. medium was added and the tube shaken at 225 rpm for 1 hr at 37°C for expression. The tube was then gently vortexed and 100 µl of neat or 1 in 10 dilution (in S.O.C.) of reaction was spread onto LB plates with 100 µg/ml ampicillin. The plates were incubated overnight at 37°C. Five colonies were picked by gentle scraping with a sterile pipette-tip and were used to inoculate 10 ml of LB broth containing 100 µg/ml ampicillin. These cultures were incubated overnight with vigorous shaking at 37°C. Growth from these cultures was screened for the IL-8 gene (clone of interest) by using small-scale plasmid DNA preparations (minipreps) followed by restriction enzyme digestion.

2.2.12.2. Screening transformants for the IL-8 promoter

The Wizard™ minipreps DNA purification system (Promega) was used as per the manufacture's protocol. A 2 ml aliquot of culture medium was centrifuged at 12,000g and the cell pellet resuspended in cell resuspension solution (Tris-HCl 50 mM, pH 7.5; EDTA 10 mM; RNase A 100 µg/ml) and transferred to a fresh 2 ml tube. 200 µl of cell lysis solution (NaOH 0.2 M, SDS 1%) was added and mixed by inversion, resulting in a clear solution. Two hundred µl of neutalisation solution (potassium acetate 1.32 M) was next added and the tube again inverted several times to allow mixing. Following spinning at 12000g for 5 min the clear supernatant as decanted to a new tube. One ml of purification resin (well mixed) was added to the supernatant and mixed by inversion. This solution was then transferred into 3 ml syringe onto which a minicolumn has been attached and gently pushed through the column with the plunger. The syringe was the detached and filled with 2 ml column wash (NaCl 200 mM, Tris-HCl 20 mM, EDTA 5 mM all diluted 1:1.4 with 95% ethanol) which was then gently pushed through the minicolumn. The minicolumn was then inserted into a 1.5 ml tube and spun at 12,000g for 2 min. The DNA was

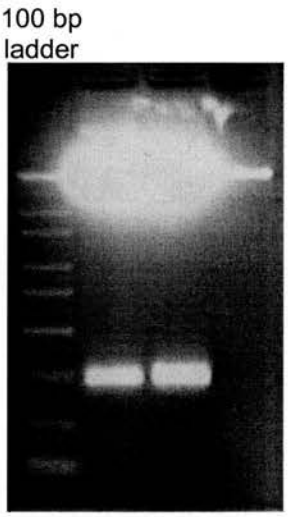
then eluted by transferring the column to a fresh tube, adding 50 µl of warm (70°C) TE buffer and allowing to stand for 1 min before spinning at 12,000g for 20 sec. The concentration of DNA eluted was measured by UV spectrophotometric measurement of the O.D. at 260 nm.

Purified plasmid DNA was digested with *Pst* 1 and *Xba* 1 restriction enzymes in the following reaction mixture:

DNA 0.12 µ/µl	20 µl
Autoclaved MiliQ water	21.6 µl
Buffer D (10x)	4 µl
BSA (10 mg/ml)	0.4 µl
Pst 1 (10 U/µl)	2 µl
Xba 1 (10 U/µl)	2 µl
	40 µl Total

Digestion was performed for 1 hr at 37°C. Twenty µl of digestion mix was run on a 1.2 % agarose gel in 1x TAE buffer. A successful digestion of a plasmid containing the IL-8 promoter would be expected to yield a 280 bp product based on the known restriction sites. **Figure 2.2.13.2** demonstrates this in 2 successful clones. One successful clone was subsequently used in a large-scale culture and DNA preparation (method similar to that described for mini-preps above) to yield sufficient quantities of IL-8 promoter for transfection experiments.

Figure 2.2.12.2



Legend for Figure 2.2.13.2 IL-8 promoter cut from transformed plasmid.
A 280 bp product, representing the IL-8 wild-type promoter was cut from 2 successfully transformed pCAT basic plasmids

2.2.12.3 Protocol for liposome-mediated cell transfection.

This protocol is adapted from that described in the 'Transfection Guide' published by Promega (Promega, Southampton, UK) and was applied to transfection experiments in CHO-K1, the CHO-K1 HIF-1 expressing (C4.5) and non-expressing (Ka13) variants, and human monocyte derived macrophages.

Tfx™-10 reagent (3.1 mg) was allowed to equilibrate to room temperature and dissolved in 400 µl of nuclease-free water (1 mM final concentration of the cationic lipid component). The sample was vigorously vortexed and then placed in a water bath at 65°C for 1 min. The reagent was then stored at -20°C until use (stable for up to 8 weeks). Transfections were performed in 6 well plates when cells were 70-80% confluent. Seeding CHO-K1 or C4.5 cells at $2x10^5$ cells per well and Ka13 cells at $3x10^5$ cells per well in 3 ml medium supplemented with 10% FCS resulted in 70-80% confluency over 2-3 days. Human monocyte derived macrophages were cultured as described in **2.2.1.1**. The transfection parameters used were 2.5 µg total DNA per well with a Tfx:DNA ratio of 2:1 in 1 ml of serum-free (SF) medium (studies described in Chapter 6 showed this to be the optimum ratio for transfection efficiency in CHO cells. The constituents of a transfection mixture for 1 well were:

IL-8 or pCAT control DNA	2 μg
pSV-β-Gal DNA	0.5 μg
Tfx™-10	7.5 μl
SF medium (to final volume)	1 ml

The DNA was first added to warm SF medium and vortexed. The Tfx™-10 reagent was then added, the mixture vortexed, and incubated for 15 min. Supernatants from cultured cells were carefully removed and 1 ml of transfection mixture (vortexed) per well overlain. Transfection was performed 37°C for 1 hr, following which 1 ml pre-warmed medium supplemented with 20% FCS was added to each well. The cells were treated with normoxia, hypoxia, CoCl (100 μM), DFO (100 μm) or PMA (100 nM) for 18 hr.

2.2.12.4. Cell lysis and ELISA for CAT and β-Gal protein

Cell lysis and ELISA's were performed using the appropriate kits (Boehringer Mannheim) as per the manufacturers protocol. Briefly, the supernatant from the treated cells was removed and the cells washed with cold PBS. One ml of lysis buffer was added to each well and the plates incubated at RT for 30 min. The lysis buffer (containing all cytoplasmic and nucleoplasmic proteins) was then gently aspirated and centrifuged at 12,000g for 15 min at 4°C to remove cell debris. Samples were aliquoted and stored at –70°C until analysis.

Total protein was measured using the BCA protein assay (Pierce, IL, USA). The CAT and β-Gal ELISA's are based on the sandwich ELISA principle. Samples (undiluted for CAT, diluted 1 in 10 for β-Gal estimation) or standards were added to wells pre-coated with anti-CAT or anti-β-Gal antibody. Following incubation for 1 hr at 37°C, the wells were washed and a digoxigenin-labelled antibody to CAT or β-Gal added. After further incubation for 1 hr and washing, the peroxidase substrate, ABTS® was added and absorbance measured at 405 nm on a plate reader. A

calibration curve was plotted from the standards and sample values calculated by extrapolation from the linear portion of this curve.

2.3. METHODS USED FOR *IN VIVO* MODEL OF ACUTE LUNG INJURY

2.3.1 Animal sedation, anaesthetic and intravascular monitoring

Female New Zealand rabbits weighing 2.8 - 3.5 kg (Charles River Ltd. Margate, Kent) were sedated with intramuscular fentanyl citrate and fluanisone (Hypnorm® 0.3 ml/kg bolus followed by 0.3 ml/kg/h) then anaesthetised with intravenous (i.v.) midazolam (Hypnovel® @ 2 mg/ml, 0.75 ml/kg) via marginal ear vein. The animals were placed in the prone position and temperature regulated by rectal thermistor and a heating blanket beneath the animal. A 22-gauge intravenous cannula was secured in both marginal ear veins for fluid and drug administration. A tracheotomy was performed and a purpose-made endotracheal tube inserted and secured. A size 3F cannula, primed with saline/10 U./ml. heparin, was inserted and tied into the right femoral artery then attached to a heart rate and blood pressure monitor (Model VT-15, Weco, UK).

A multiprobe sensor (Paratrend 7, Diametrics Ltd., High Wycombe, UK) was inserted via an introducer into the left femoral artery and advanced toward the iliac artery. This provided a continuous digital and graphical readout of arterial PCO_2, PO_2, pH and temperature. Prior to ventilation, the animals were paralysed with i.v. pancuronium bromide (Pavulon®, 0.45 mg/kg bolus then 0.3 mg/kg/h, Organon Labs. Ltd., Cambridge). Saline was infused subcutaneously at 2.5 ml/hr throughout the experiment. The animals were ventilated with a pressure-controlled respirator (Model RSP1002, Kent Scientific Corp.), the initial settings being 30 bpm, 12.5 cm. H_2O for max. inspiratory pressure and zero end-expiratory pressure. The ventilator inlet was connected to a 15 litre gas reservoir initially supplied with room air and animals allowed to equilibrate with this system for 30 min prior to instillation of

acid. Fifteen minutes prior to acid-instillation, the animals were injected with 4 ml of Evans blue-labelled albumin (0.25% Evans blue, 4% BSA) intravenously.

2.3.2. Localised bronchoscopic instillation of hydrochloric acid

HCl pH 1.5 (~0.05 M) was prepared by adding HCl to $^1/_3$ normal saline as previously described (Folkesson et al., 1995). A customised T-piece, which permitted the animal to be ventilated whilst being bronchoscoped, was interconnected between the endotracheal tube and the ventilator. The tip of a neonatal bronchoscope (Olympus BF type 3C10) was wedged under direct vision into the left lower lobe of the animal. A pre-measured length of nylon tubing (0.94 mm o.d., Portex Ltd., Hythe, Kent) was threaded down the instrument port of the bronchoscope until its tip could be viewed emerging distally from the bronchoscope, HCl (1.0 ml/kg) was then slowly injected (over 2 minutes) through the nylon tube into the lobe, followed by a bolus of air to ensure complete delivery of the acid. The nylon tubing and the bronchoscope were withdrawn and the direct connection of the ventilator to the animal restored.

2.3.4. Ventilation strategy and induction of hypoxia, reoxygenation and hyperoxia

All animals were ventilated for 4 hours following acid instillation. The 'normoxic' animals received air and minor adjustments to ventilation parameters were made in order to maintain an arterial PO_2 of 11 KPa (acceptable range range 9.5 - 12 KPa), PCO_2 of 4.3 KPa (acceptable range 3.8 - 5.0 KPa) and a pH of 7.5 (acceptable range 7.38 - 7.6). This was achieved with a respiratory rate of 38 bpm (range 33–44) and a peak inspiratory pressure of 13 cm H2O (acceptable range 11 - 16). In the hypoxic / hyperoxic group, animals received a gas mixture of 83% N / 17% O_2 for 2 hours to induce hypoxia (PO_2 of 5 KPa, acceptable range 4.0-5.5 KPa) followed by 100% O_2 for 2 hours to induce hyperoxia (PO_2 60 KPa, acceptable range 52 – 70 KPa). Minor adjustments were made to the ventilation parameters in order to maintain normal PCO_2 and pH. In the hypoxic / controlled reoxygenation group, animals received a gas mixture of 83% N / 17% O_2 for 2 hours to induce hypoxia as above, followed by

a gas mixture of 70% N_2 / 30% O_2 for 2 hours to achieve controlled reoxygenation (PO_2 15 KPa, acceptable range 13-17 KPa). All animals received a 3-second 'sigh' (peak inspiratory pressure increased to 22 cm H_2O) every 30 min during ventilation to prevent atelectasis. The overall protocol for the animal studies is shown in **Figure 2.3.4 A.** A representative print-out obtained from continuous monitoring of PO_2, PCO_2 and pH in an animals ventilated under hypoxic / hyperoxic conditions (**Figure 2.3.4B**)

Figure 2.3.4 A

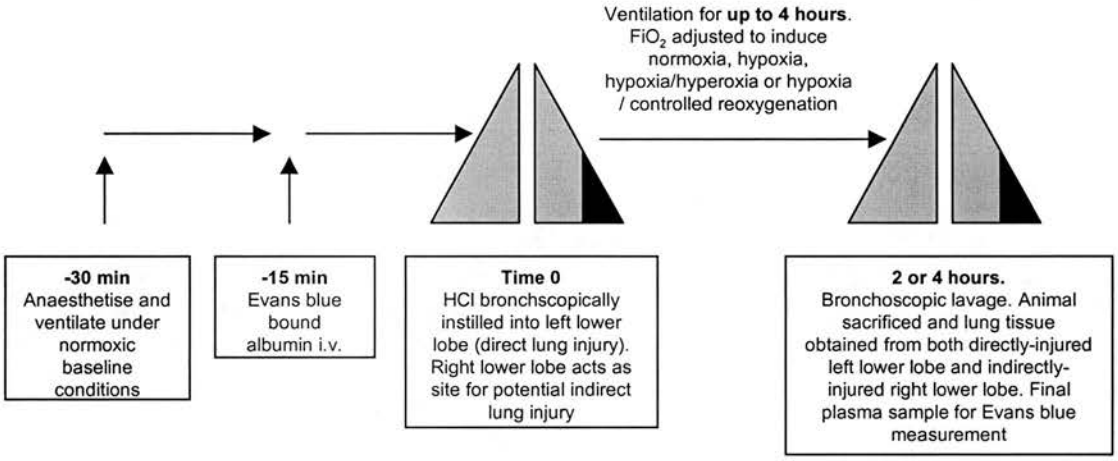
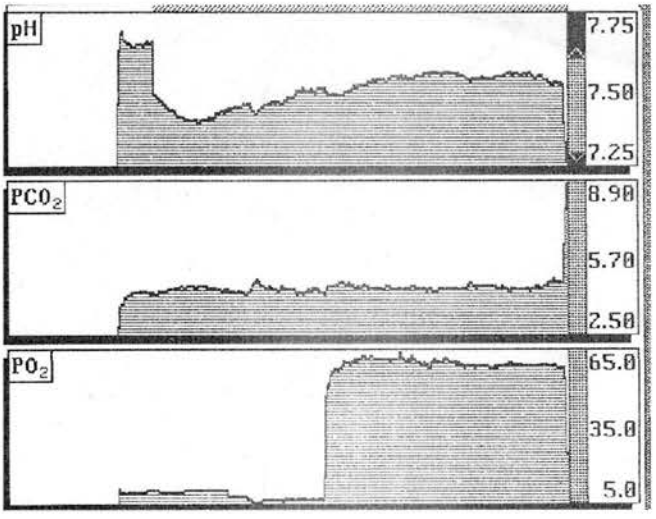


Figure 2.3.4.B



Legend for figure 2.3.4.A and B. The left lower lobe in anaesthetised, ventilated rabbits was instilled with HCl. Arterial oxygenation in the ventilated animal was manipulated by altering FiO_2 only. The Paratrend sensor allows continuous monitoring of arterial pH, PCO_2 and PO_2. Animals could be ventilated for up to 2 hours under hypoxic or hyperoxic conditions without significantly influencing PCO_2 or pH. The trace shown is from an animal ventilated under hypoxic / hyperoxic conditions

2.3.5. Bronchoalveolar lavage (BAL) and lung excision.

Both the left lower lobe (acid instilled) and right lower lobe (no acid) were lavaged bronchoscopically in animals. The bronchoscope was advanced through the T-piece and endotracheal tube and its tip steered then wedged into the appropriate lobe. Ten ml of sterile saline (4°C) was injected via the appropriate channel into the lobe and allowed to stand for 2 minutes. Lavage fluid was gently aspirated back through the bronchoscope and a further 5 ml of saline was similarly instilled and lavaged. The total volume of lavage fluid recovered using this technique was 11.5 ml (range 10.5 – 12.2 ml). BAL fluid was centrfiuged at 1500 rpm (600 g) for 5 min at 4°C to recover cells. Total cell counts were performed with a haemocytometer. Samples of cells were pelleted onto glass slides with a Cytospin 2 (Shandon Scientific, Cheshire, UK) and stained with Diff-Quick (Oberckochen, Germany), a modified Wright-Giemsa stain. Differential counts were made by counting 500 cells under oil immersion (x 100). The BAL supernatant was recentrifuged at 1000 g for 10 min at 4°C to remove cellular debris and used to determine protein leak and IL-8 level as described below.

Immediately following lavage the animal was euthanased with sodium pentabarbitone (Sagatal, 200 mg/ml, Rhone Merieux Ltd., Harlow, Essex) and both lungs excised and placed on ice. Pieces of both left and right lower lobes were snap frozen in liquid nitrogen until use.

2.3.6. Lung tissue homogenisation for protein and RNA

Lung homogenisation was performed using a Polytron® homogeniser (Kinematica, Switzerland). For tissue to be used in protein assay and IL-8 ELISA, lung pieces (~300 mg) were homogenised from frozen at 20,000 rpm for 30 seconds in a buffer containing PBS, protease inhibitor cocktail (Complete™ Boehringer Mannheim) and 0.05 % Nonidet P40. The sample was the spun at 1000 g for 10 min at 4°C and the supernatant assayed for total protein content and IL-8 concentration by Pierce assay and ELISA respectively. For RNA extraction, lung tissue (~150 mg) was homogenised from frozen in 3 ml TRIzol® reagent at 20,000 rpm for 45 seconds.

The sample was then spun at 500 g for 5 min at 4° C to remove debris. The supernatant was then processed as per the manufacturers' protocol, based on the method of Chomczynski and Sacchi, (1987). Total RNA was dissolved in DEPC–treated water and stored at –70° C until use.

2.3.7. Measurement of lung protein leak

Leakage of Evans blue-labelled albumin in the bronchoalveolar lavage fluid was used to determine lung protein leak, a modification of a previously described method (Patterson et al., 1992). An aliquot of BAL fluid was taken and the optical density at 620 nM measured by spectrophotometry (P8700, Philips). This result was converted into a measure of albumin content using a pre-determined standard curve and compared with that obtained from blood plasma (obtained at the time of lavage). The degree of lung leak is expressed as 'plasma equivalents', that is the amount of plasma that would have been required to pass into the alveolar space to account for the presence of Evans blue-labelled albumin in lavage fluid. This method has been found to be as sensitive as using ^{125}I-labelled albumin leak (Patterson et al., 1992), and has been used as a measure of lung protein leak in several animal studies (Mason and Effros, 1983; Rogers et al., 1989).

2.3.8. Rabbit IL-8 ELISA

Rabbit IL-8 ELISA was performed on BAL fluid and lung tissue homogenate using reagents kindly supplied by Dr Caroline Hébert (Genentech Inc., CA) and using minor modifications of a previously described method (Folkesson et al., 1995). Microtitre plates (96-well; Costar, Cambridge, MA) were coated overnight at RT with a mouse anti-human IL-8 monoclonal antibody (ARIL8.2; final concentration 2 µg/ml, diluted in 0.5% BSA / 0.05% Tween 20 / PBS). Wells were washed 3 times with was buffer (0.05% Tween 20 in PBS) and non-specific binding sites blocked for 1 hour with 200 µl of 1% BSA in PBS. Rabbit recombinant IL-8 (rIL-8) and samples (BAL fluid or lung homogenate, both without dilution) were added to the wells and incubated at RT for 2 hrs. After washing, the secondary antibody (biotinylated

8C1.1.6; 1 in 3500 in 0.5% BSA / 0.05% Tween 20 / PBS) was added and following a further 2 hr incubation and subsequent wash, horseradish peroxidase – conjugated streptavidin (1:20,000) was added for 30 min. Substrate solution consisting of tetramethyl benzidine 100 μg/ml, 4.5 mM H_20 in 0.1 M sodium acetate-citrate pH 4.9 was added and the plates incubated in darkness at room temperature for 30 min. The reaction was stopped with 50 μl of 0.5 M H_2S0_4.

Plates were read at 450 nm in an plate reader (Dynatech MR5000). Sample values were determined using a software package (Assayzap) from a standard curve. The linear range was ~320-7500 pg/ml, and the lower limit of detection 60 pg/ml. Despite measuring rabbit samples undiluted, IL-8 levels tended to fall below the linear range. However, the sensitivity of the assay was sufficient to allow reproducible measurements. For lung homogenate samples, IL-8 results were corrected for total protein, measured by BCA protein assay and expressed in pg/ml/mg total protein. As BAL volumes were consistent with minimal variability (median 11.5, range 10.5 – 12.2 ml), no correction was applied and the results expressed in pg/ml.

2.3.9. Semi-quantitative rabbit IL-8 RT-PCR

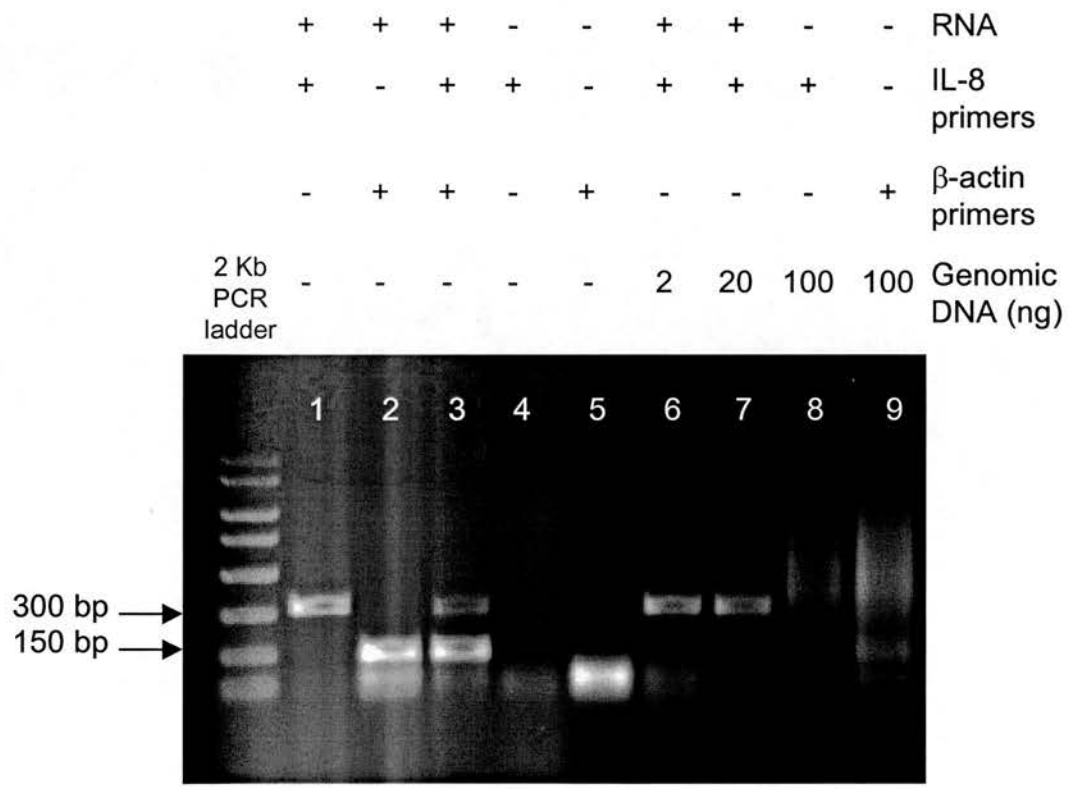
IL-8 mRNA levels in lung tissue were determined by semi-quantitative RT-PCR from total RNA derived from lung homogenates as described. First strand cDNA was synthesised from 1 μg total RNA in a reaction mix containing 0.5 μg Oligo-dT primer, 1 mM dNTPs, 16U RNasin, 20U AMV-RT and 10 mM MgCl. The reaction mix was incubated for 45 min at 48°C. Rabbit IL-8 primers, designed from the GenBank succession sequence number M57439 (Yoshimura et al., 1991) were; **5'-ATG AAC TCC AAG CTG GCT-3'** (sense) and **5'-TTA TGA CTC TTG CTG CTC AGC-3'** (antisense). These primers reside respectively in exon 2 and exon 4 of the rabbit IL-8 gene, and hence span 2 introns. The predicted and observed product size for rabbit IL-8 using these primers was 308 bp.

PCR conditions were: 94°C for 1 min (denaturation), 55°C for 1 min (annealing) and 72°C for 1 min (extension) followed by a final 5 min extension at 72°C. The human

β-actin gene was employed as a housekeeping product. The primers used were: **5'-CCA CCA ACT GGG ACG ACA TG-3'** (sense) and **5'- GTC TCA AAC ATG ATC TGG GTC ATC-3'** (antisense). The PCR conditions for β-actin were: 94°C for 1 min (denaturation), 60°C for 1 min (annealing) and 72°C for 1 min (extension) followed by a final 5 min extension at 72°C. The predicted product size, based on human studies, was 180 bp.

A series of experiments were performed to validate and refine the rabbit IL-8 and β-actin RT-PCR (**Figure 2.3.9A**). These included an experiment to define the linear phase for PCR product versus cycle number (**Figure 2.3.9B**), and based on these results, 26 cycles were used in subsequent RT-PCR experiments.

Figure 2.3.9A

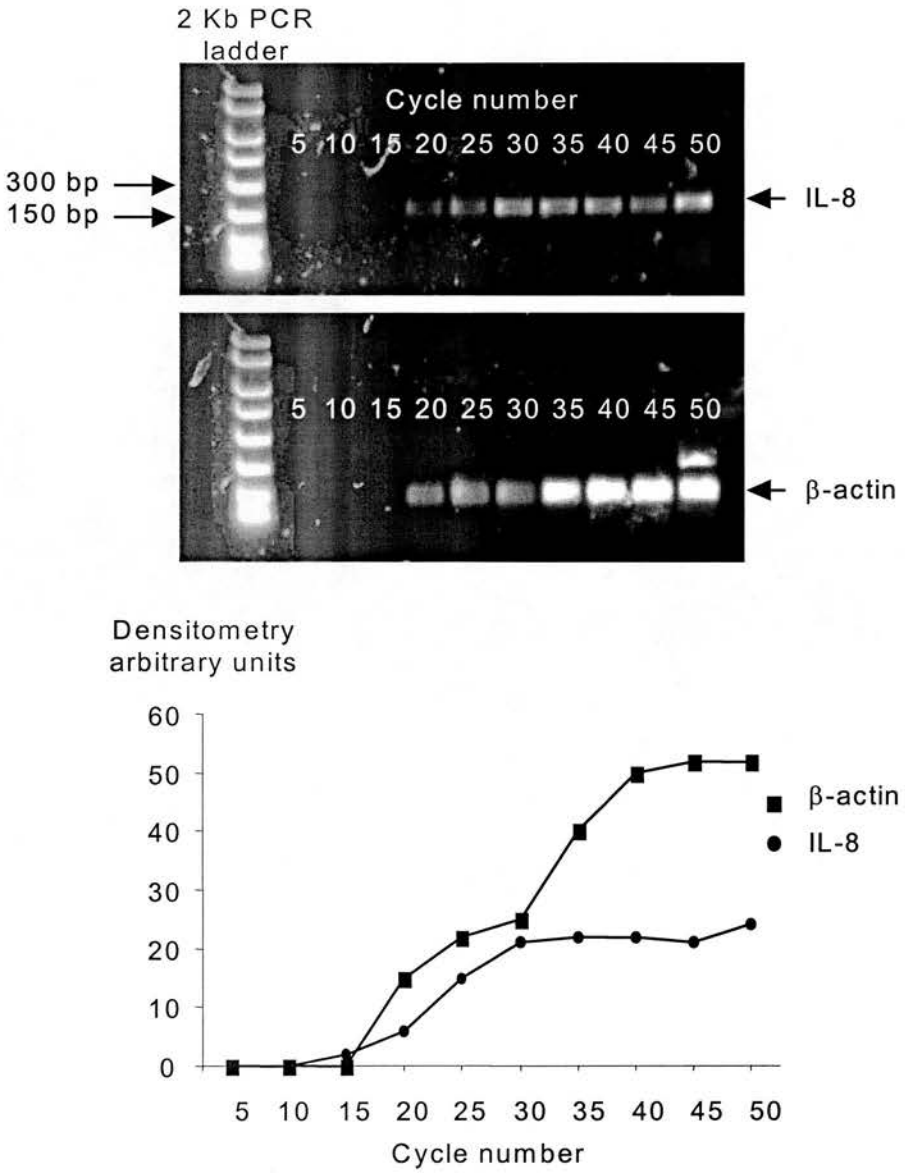


Legend for Figure 2.3.9A. Validation of Rabbit IL-8 RT-PCR
A series of control experiments were performed to validate the rabbit RT-PCR. Total RNA from rabbit alveolar macrophages stimulated for 4 hours with LPS (500 ng/ml) was used in RT-PCR reactions, using reagents described in text. Lanes 1 and 2 show

Legend for Figure 2.3.9A continued

a 308 bp and 180 bp IL-8 and β-actin PCR product respectively. In Lane 3, both IL-8 and β-actin primers were used in the same reaction ('multiplex-PCR'). Although the bands are distinguishable, the intensity of both is reduced. Lanes 4 and 5 represent IL-8 and β-actin primers respectively in the absence of RNA. The visible LMW bands represent primer pairs. Although IL-8 and β-actin primers were designed to span at least one intron, additional controls were performed to ensure the PCR products did not represent amplified genomic rabbit DNA. In Lanes 6 and 7, the IL-8 RT-PCR reaction was 'spiked' with 2 ng and 20 ng of genomic rabbit DNA respectively. The observed 308 bp IL-8 product was not affected. In Lanes 8 and 9, 100 ng of genomic rabbit DNA was amplified with IL-8 and β-actin primers in the absence of RNA or RT-AMV. A smear is seen, demonstrating that neither IL-8 nor β-actin can be resolved by PCR of genomic DNA.

Figure 2.3.9B



Legend for Figure 2.3.9.B Cycle number versus product for rabbit IL-8 and β-actin RT-PCR. Rabbit alveolar macrophages were stimulated with LPS 500 ng/ml.

Legend for Figure 2.3.9B continued
Total RNA was extracted and RT-PCR performed using primers and conditions described in text. Product increases exponentially with increasing cycle number for both IL-8 and β-actin. 26 cycles falls within the linear portion for both PCR products, and this cycle number was used for measuring rabbit IL-8 mRNA expression by RT-PCR in subsequent experiments. The PCR gels (**top**) and densitometry (**lower**) from n=1 experiment are shown.

2.4. STATISTICAL ANALYSIS

For *in vitro* experiments, results are reported as pooled data from n separate experiments performed in triplicate unless otherwise stated, where n=1 refers to an individual donor, in which each treatment condition is replicated 3 times. The gel images shown are representative of n≥3 experiments. For *in vivo* experiments, results are reported as pooled data from n separate experiments, where n=1 refers to an individual animal. Data is presented as mean ± SEM unless otherwise stated. Statistical analysis was by Student's t-test after assessing for equality of variance between groups.

CHAPTER 3

IN VITRO STUDIES OF ACUTE HYPOXIA, HYPEROXIA AND STRESS MEDIATORS ON IL-8 GENERATION IN MACROPHAGES

3.1. INTRODUCTION

It is a well-recognised phenomenon that only a subgroup of patients with major predisposing insults, such as multiple trauma, sepsis, aspiration or pancreatitis, develop the catastrophic inflammatory response characteristic of acute respiratory distress syndrome (ARDS) (Fowler et al., 1983). Our group has previously demonstrated that raised intrapulmonary IL-8 levels are associated with progression to ARDS (Donnelly et al., 1993; Hirani et al., 2001). In patients with major trauma, these raised levels of IL-8 were detectable in bronchoalveolar lavage (BAL) fluid within a few hours of the initiating event (median 95 minutes, range 30-240 min), well before clinical evidence of ARDS. Immunohistochemical analysis has implicated the alveolar macrophage as a potent source of IL-8. The mechanisms by which intrapulmonary IL-8 may be upregulated in this context are unknown.

The immediate aftermath of a major trauma event is characterised by a constellation of rapid and often profound physiological and metabolic events. Hypotension, hypoxaemia, tissue hypoxia and acidosis are frequently exhibited. Activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system generates both systemic and localised release of catecholamines (Elenkov et al., 2000) and neuro-endocrine mediators. Circulating and tissue levels of archetypal early proinflammatory cytokines such as IL-1, IL-6 and TNF-α may be raised and generate a 'cytokine cascade'. Endotoxin has been implicated as a potential 'hit' post- trauma, with the suggestion that commensal bacteria are able to translocate from compromised gut into the circulation during a period of hypovolaemic shock (Baker et al., 1988; Rush et al., 1988). Furthermore, the resuscitation phase following trauma introduces additional potential hits in the form of reperfusion, reoxygenation, hyperoxia, and ventilation-induced barotrauma (Saadia, 1996; Rocker, 1997; Czermak et al., 1999; Dos Santos and Slutsky, 2000).

In the context of a vastly complex stress response, I have focused on a selection of potential inflammatory stimuli to which the lung may be exposed following major trauma: 1) hypoxia, a consequence of several factors including hypoventilation, atelectasis, and contusion. Our clinical study had revealed a relationship between

impaired gas exchange, as manifest by reduced PaO_2 / FiO_2, and raised IL-8 ($r = -0.56$, $P<0.001$, $n=42$) (Hirani et al., 2001); 2) hyperoxia, which is relevant in the immediate resuscitation phase of trauma, during which high flow oxygen is delivered to the ventilated lung; 3) the stress mediators adrenalin, substance P and MIF, and the archetypal proinflammatory mediators endotoxin, TNF-α and IL-1β.

The aim of this study was to determine the *in vitro* effects of these potential stimuli in human monocyte-derived macrophages. Based on our clinical observations, particular emphasis was placed on the acute *in vitro* effects of hypoxia and hyperoxia.

3.2. RESULTS

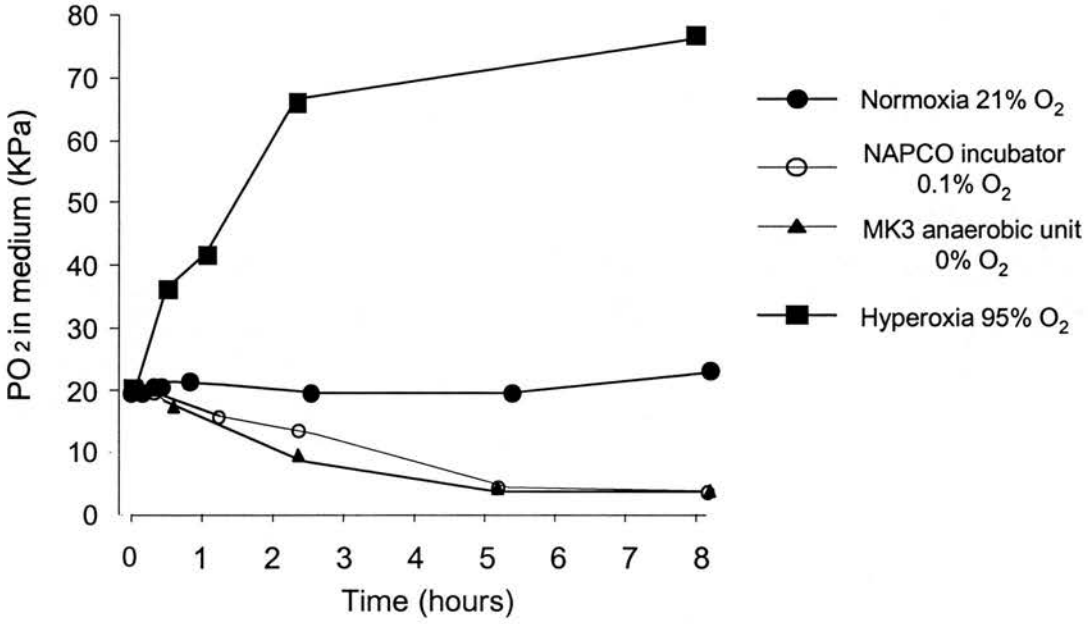
3.2.1. Rate of equilibration of medium without cells with environmental oxygen under hypoxic or hyperoxic conditions.

One of the key aims of this study was to determine the effects of *acute* hypoxia and hyperoxia on cultured cells. It was therefore important to determine the time required for culture medium to equilibrate with changes in environmental oxygen. In initial studies medium with 2% serum, without cells, was aliquoted into 35 mm (6-well) plates, 2.5 ml per well, and incubated in a normoxic incubator (PO_2 21%, PCO_2 5%), the NAPCO incubator (PO_2 0.1%, PCO_2 5%), the MK3 anaerobic unit (PCO_2 10%, H_2 10%, N_2 80%) or a hyperoxic chamber (PO_2 95%, PCO_2 5%). The plate lids were left on during the incubations. The partial pressure of dissolved oxygen was measured at intervals with an OXEL-1 oxygen probe, the tip placed ~5 mm above the base of the plate. For incubations performed in the MK3 anaerobic unit, these measurements were made without removing the plates from the chamber. This technique was not feasible for incubations performed in the normoxic, NAPCO hypoxic or hyperoxic conditions. In these cases, the plates were removed from the incubator and the PO_2 in the medium immediately measured. The results are shown in **Figure 3.2.1**

3.2.2. Effect of cultured macrophages on PO_2 of medium in hypoxic conditions and following reoxygenation

The studies of oxygen equilibration under hypoxic and hyperoxic conditions were performed in medium with 2% serum without cells. Under severely hypoxic conditions (0% and 0.1% oxygen), the equilibrated PO_2 in solution was ~3.8 KPa, as measured by OXEL-1 probe. However, it is likely that the presence of actively metabolising cells would influence medium oxygenation. To explore this further, mature human monocyte-derived macrophages cultured in 6-well plates were transferred from a normoxic incubator to the MK3 anaerobic chamber. The overlying culture medium was removed and replaced with 2.5 ml of medium with 2% serum, which had been pre-incubated in the anaerobic unit for 16 hours. The PO_2 of the medium was measured at intervals, ~5 mm

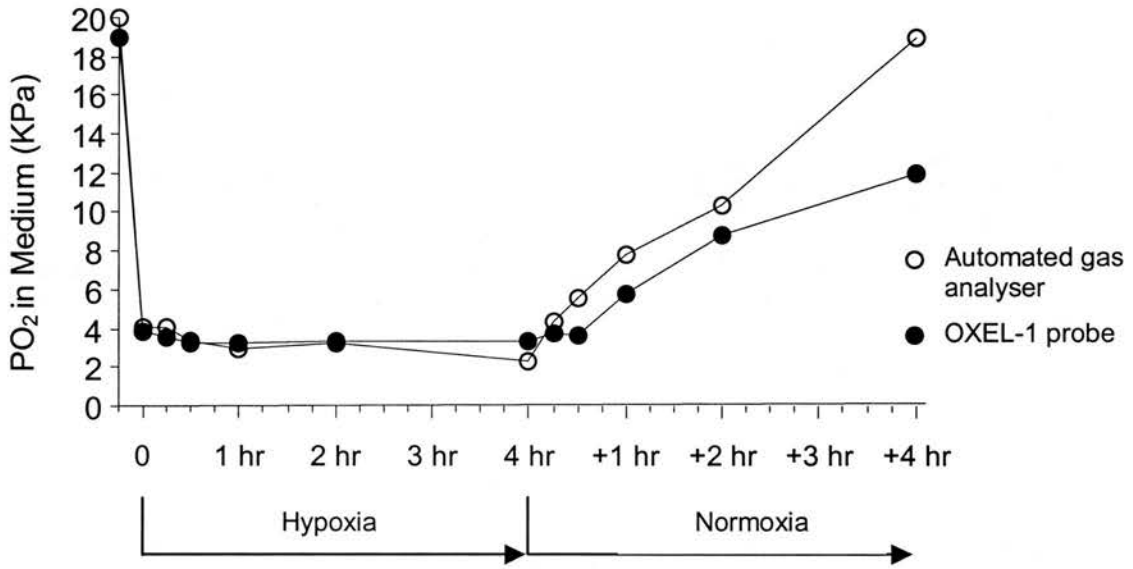
Figure 3.2.1



Legend for Figure 3.2.1. Partial pressure of oxygen in medium without cells incubated in normoxic, hypoxic or hyperoxic conditions over time.
The PO_2 in medium was measured by OXEL-1 probe placed 5mm above the bottom of 6-well plate containing 2.5 ml of medium with 2% serum. Under these conditions, equilibration with a 0% and 0.1% atmosphere was achieved by approximately 5 hours (~3.8 KPa). Equilibration in the hyperoxic chambers approached equilibration by 2 hours (~ 70 KPa). The data shown is from n=1 experiment. The experiment was repeated once, with very similar results (data not shown).

above the cells with the OXEL-1 probe. In addition, because of the possibility protein may foul the OXEL-1 probe tip in the presence of cells, PO_2 was also measured by automated gas analyser (model ABL-330, Radiometer Copenhagen, see Chapter 2). After 4 hours, the plates were removed from the anaerobic chamber, returned to the normoxic incubator and the PO_2 of medium again measured at intervals. The results are presented in **Figure. 3.2.2.** The PO_2 in hypoxia-equilibrated medium fell slightly within 15 min of transfer onto macrophages, and reached an apparent equilibrium by 30 min at $PO_2 \sim 3.5$ KPa. Re-exposure to a normoxic environment resulted in a slow reoxygenation of the medium, such that the PO_2 value was ~ 6 KPa by 1 hour and ~12 KPa by 4 hours, as measured by OXEL-1 probe. Measurement of PO_2 by automated gas analysis yielded similar results, except at 4 hours post-reoxygenation, a time-point at which PO_2 measured by OXEL-1 probe was consistently lower than that measured by automated gas analyser.

Figure 3.2.2



Legend for Figure 3.2.2. Partial pressure of oxygen in medium in the presence of macrophages under hypoxic conditions, and following re-exposure to normoxia. Monocyte-derived macrophages were transferred from a normoxic incubator to the MK3 anaerobic chamber, and the overlying medium replaced with medium with 2% serum pre-incubated in the anaerobic chamber (Time 0). At 4 hours, the culture plates were transferred back to a normoxic incubator. Automated gas analyser or OXEL-1 probe was used to measure PO_2 in medium at intervals. The data-points represent the mean from n=2 separate experiments.

3.2.3. The effect of varying oxygenation on pH of medium

Since the partial pressure of CO_2 also varied between the normoxic, NAPCO, MK3 and hyperoxic incubations, the pH of the medium in the presence of cells was also measured after 4 hours incubation. An automated gas analyser was used, which in addition to pH, also measured dissolved oxygen and carbon dioxide (**Table 3.2.3**). Sterile PBS and H_2O were also incubated in the MK3 anaerobic chamber. Although PCO_2 levels were higher in medium cultured in the anaerobic unit, pH was not greatly affected, presumably reflecting the buffering capacity of medium. The effects of severe acute hypoxia and hyperoxia on glucose concentration in medium was also assessed (Randox glucose assay, kindly

performed by Clinical Biochemistry, Edinburgh Royal Infirmary). Glucose levels were not significantly different between treatments.

Table 3.2.3

		OXEL-1 probe	Automated Gas Analyser			Glucose (mM)
		PO_2	PO_2	PCO_2	pH	
Normoxic incubator 21% O_2 / 5% CO_2	Medium / 2% serum	19.8 19.4	22.1 19.8	4.5 4.3	7.36 7.32	24.2 23.5
MK3 anaerobic unit 80% N_2 / 10% H_2 / 10% CO_2	Medium / 2% serum	3.8 3.9	4.4 4.1	6.5 6.4	7.30 7.31	23.5 24.6
	PBS	3.5 3.7	4.0 4.3	6.1 6.9	7.33 7.37	0 0
	Sterile water (no cells)	3.8 3.7	4.0 3.8	6.1 6.9	7.0 6.8	0 0
NAPCO hypoxic incubator 95% N_2 / 5% CO_2 / 0.1% O_2	Medium / 2% serum	3.9 3.5	4.4 4.7	5.3 4.9	7.32 7.3	24.2 23.6
Hyperoxic sealed chambers 95% O_2 / 5% CO_2	Medium / 2% serum	73 78	80 84	6.1 5.9	7.42 7.44	22.7 23.4

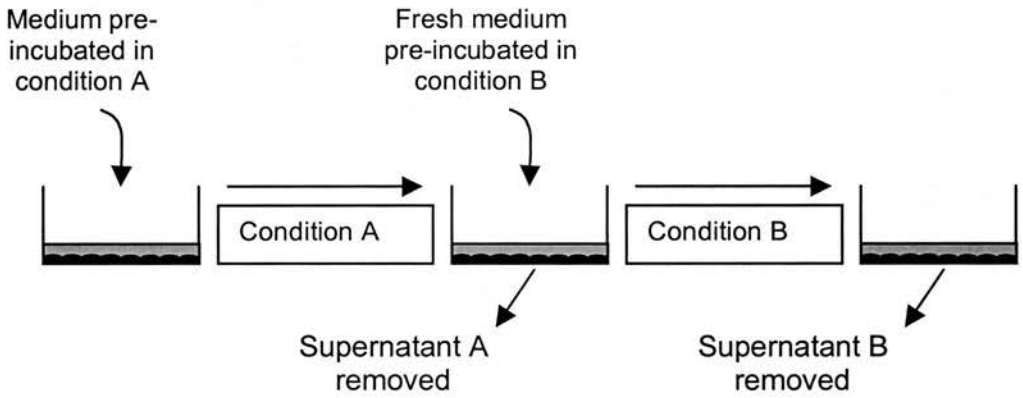
Legend for Table 3.2.3 The effects of varying gas exposures on medium Dissolved oxygen in solution in the presence of cells (except sterile water, which was without cells) was measured by OXEL-1 probe and by automated analyser. The latter also measured CO_2 and pH. All measurements were made after 4 hours incubation at 37°C. Experiments were performed twice, and both values are shown.

The studies of medium glucose levels suggest only that the effects of hypoxia and hyperoxia described later were not a result of glucose deprivation. The precise effects on cellular metabolism are not determined by this method.

The presented studies of oxygen equilibration have shown that equilibration of oxygen content in medium with environmental oxygen takes place over a period of hours. In order to expose cells to rapid changes in oxygenation, the medium therefore requires pre-incubation in the relevant environment. In all subsequent experiments, medium was aspirated from cells and replaced with pre-incubated medium to achieve rapid hypoxia,

reoxygenation or hyperoxia (**Figure 3.2.3.1**). All hypoxic experiments were performed in the MK3 anaerobic chamber.

Figure 3.2.3.1



Legend for Figure 3.2.3.1 Experimental protocol used to achieve rapid changes in oxygenation in cell culture. The studies of rate of equilibration of culture medium in varying oxygen environments suggest that rapid changes in oxygenation may only be achieved by pre-incubation of medium in the required environment. This protocol was employed for subsequent hypoxia, reoxygenation and hyperoxia experiments

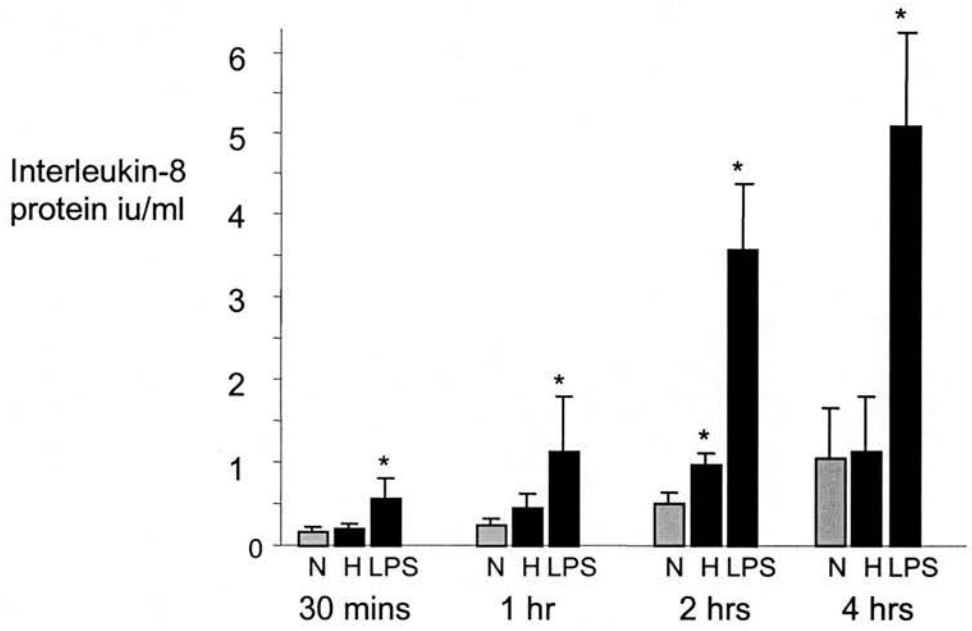
3.2.4. Viability of macrophages following hypoxia, reoxygenation and hyperoxia.

Cell viability was assessed by Trypan blue exclusion and LDH assay in cell supernatants. Under all conditions, ≥ 95% of cells excluded Trypan blue at the end of the experimental treatment. Levels of LDH were assayed in n=3 experiments. Monocyte-derived macrophages were cultured in 2% serum for 6 hours in normoxia, hypoxia or hyperoxia, or 3 hours hypoxia / 3 hours hyperoxia. LDH activity was measured by comparison with a standard curve obtained with human erythrocyte LDH solution containing 2% human serum. An LDH value of < 0.03 U was consistently obtained in cell supernatants under all conditions, compared to 23±4 U in positive controls (macrophages lysed with 0.1% Nonidet-P40).

3.2.5. IL-8 secretion from hypoxic macrophages over 4 hours

Hypoxia increased secreted IL-8 by 2 hours incubation (0.94 ± 0.18 v 0.54 ± 0.1 IU/ml, n=9, P<0.02)(**Figure 3.2.5**). At 1 hour there was a trend to increased IL-8 secretion(P=0.07, n=9). By 4 hours, there was no significant difference in IL-8 secretion (P=0.8, n=8).

Figure 3.2.5



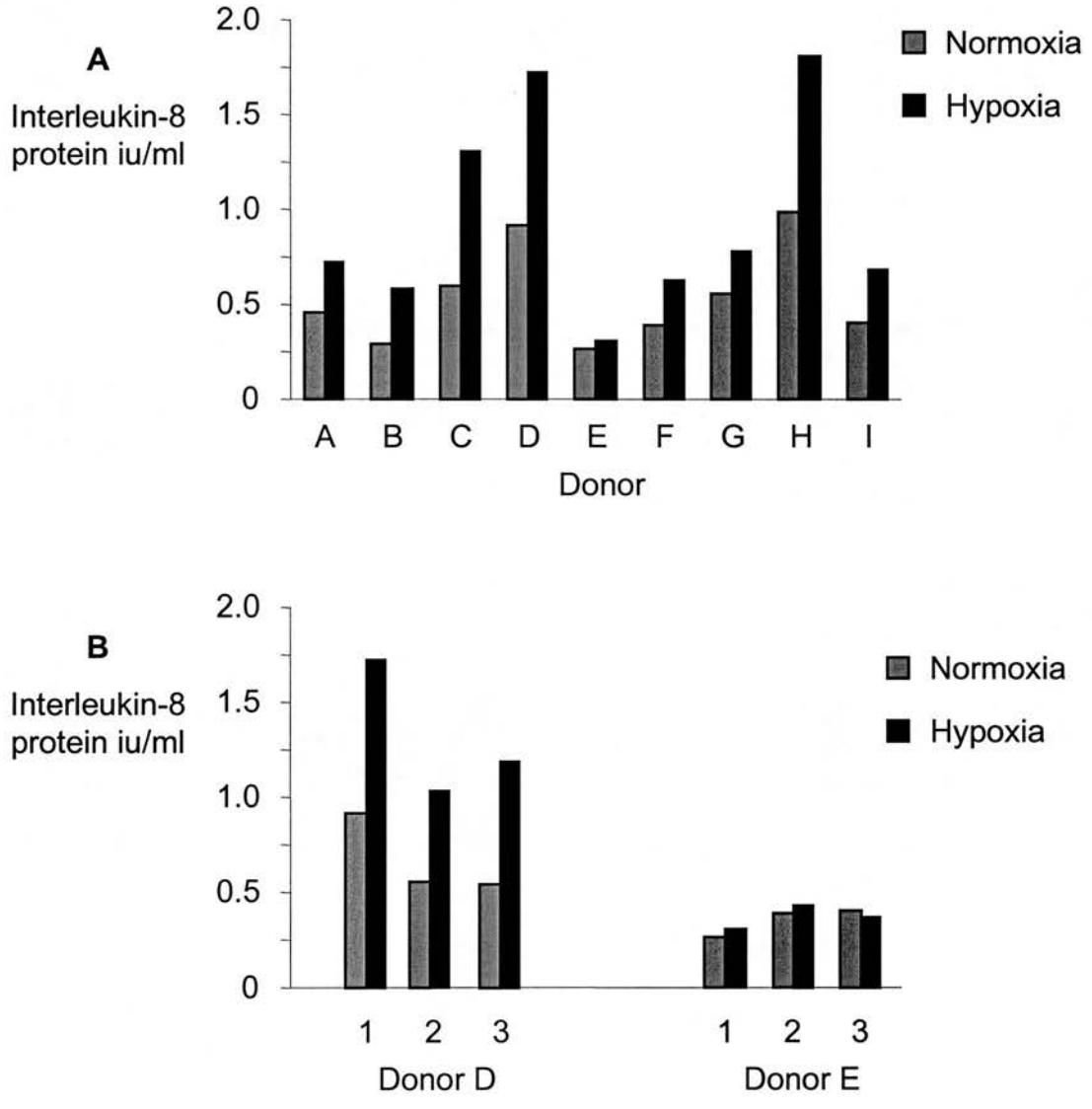
Legend for Figure 3.2.5. Interleukin-8 secretion from hypoxic and LPS treated macrophages over time. Hypoxia significantly increased IL-8 secretion at 2 hours but not at other time-points. LPS significantly increased IL-8 secretion compared to normoxia and hypoxia at all time points. Data presented as mean ± S.E.M. n=9 separate donor experiments for 2 hours. n≥8 separate donor experiments for other time-points. *P<0.02 compared to normoxic control

3.2.6. Inter- and intra-donor variability in IL-8 secretion in response to 2 hours hypoxia

The individual response to 2 hours hypoxia from macrophages derived from n=9 healthy donors is shown in **Figure 3.2.6A.** Whilst hypoxia increased IL-8 secretion from all donors, there was considerable variability in the response, from a 10 % increase (donor E) to a 120 % increase (donor C). Experiments with macrophages from donor D (80 % increase) and donor E (10 % increase) were repeated on 3 separate occasions (**Figure**

3.2.6B) Donor E demonstrated a consistent lack of effect with 2 hours hypoxia. Donor D demonstrated an increase in IL-8 release on each occasion and the magnitude of the response was similar on each occasion (80 %, 90 % and 118 % increase in experiments 1,2 and 3 respectively).

Figure 3.2.6



Legend for figure 3.2.6. Individual variation in IL-8 secretion from donor macrophages in response to 2 hours hypoxia. There was variation in hypoxia-induced IL-8 secretion between donors (A-I)(3.2.6**A**). Experiments with donor D (a high IL-8 responder) and donor E (a low IL-8 responder) were repeated on 3 separate occasions (1,2 and 3)(3.2.6**B**) with similar hypoxic responses each time.

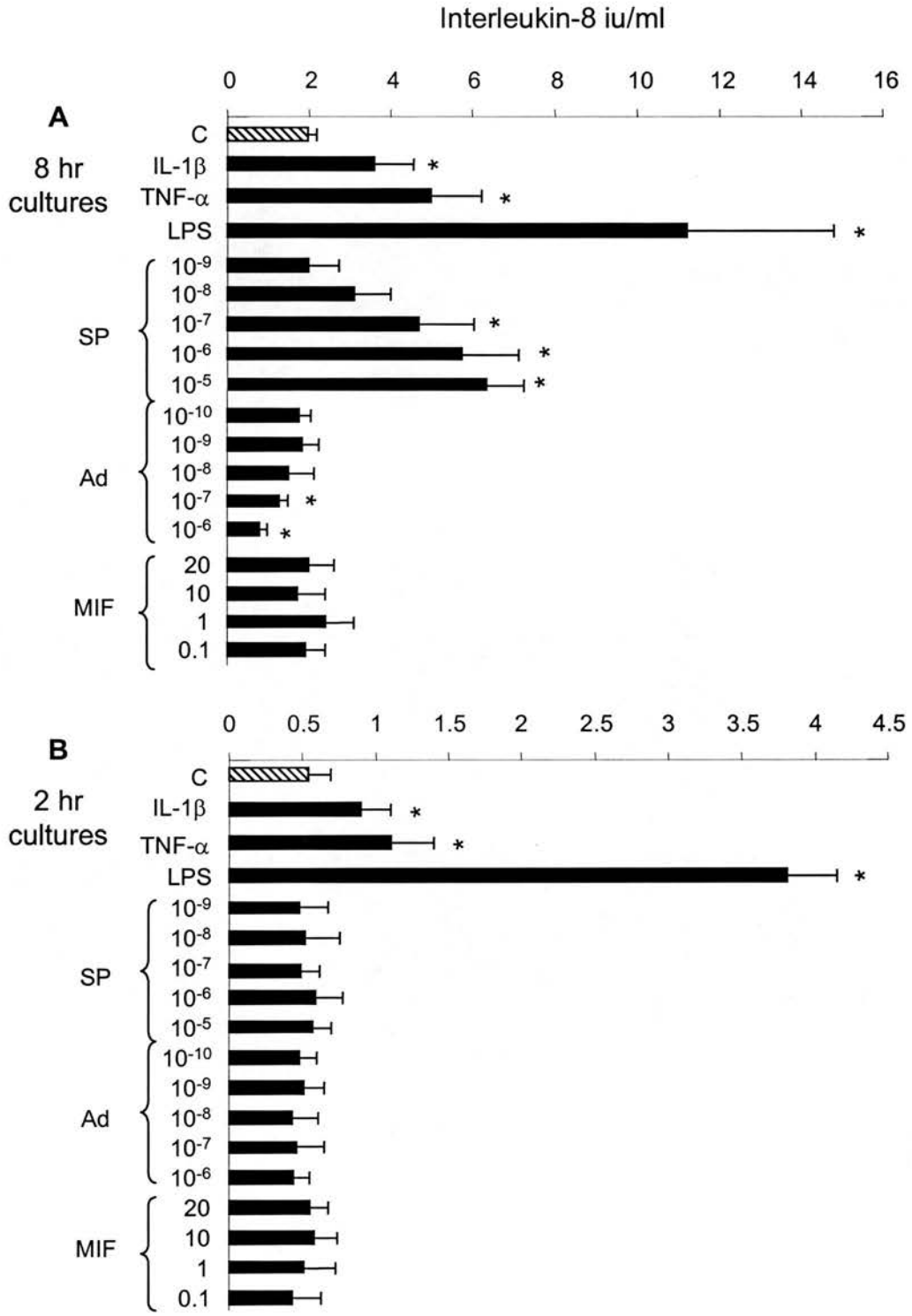
3.2.7. IL-8 secretion from macrophages treated with IL-1β, TNF-α, Substance P, adrenalin or MIF

It was apparent that the 2 hour IL-8 response to hypoxia, whilst statistically significant, was small (1.7-fold increase), particularly in comparison to that seen with LPS (**Figure 3.2.5**). Since this early time-point may be relevant with regard to the at-risk period of ARDS, the effect of other potentially clinically relevant mediators on macrophage IL-8 secretion were also studied. Monocyte-derived macrophages from healthy volunteers were treated with IL-1β, TNF -α, LPS, Substance P, adrenalin or MIF, and the IL-8 secretion into the cell supernatants after 2 and 8 hours was measured. Compared to controls, IL-1β , TNF -α, LPS and Substance P at 10^{-6} and 10^{-7} M significantly increased IL-8 secretion by 8 hours. Adrenalin 10^{-6} 10^{-7} M significantly inhibited basal IL-8 secretion compared to controls at this time-point (**Figure 3.2.7A**). By 2 hours, IL-1β, TNF-α and LPS significantly increased IL-8 secretion compared to normoxia. Substance P, adrenalin and MIF had no effect on IL-8 secretion at this early time-point (**Figure 3.2.7B**). Hence, at the early time-point, only the archetypal proinflammatory mediators significantly increased IL-8 secretion. Furthermore, the magnitude of the IL-8 response to IL-1β and TNF-α at the two hour time-point (1.6-fold increase and 2.1-fold increase respectively) were similar to that observed with hypoxia.

3.2.8. Hypoxic monocyte-derived macrophages do not store intracellular IL-8

To establish if hypoxia increased intracellular stores of IL-8, levels were measured in cultured macrophages lysed in PBS / 0.1% Nonidet P40 following careful washing in complete medium. Lysate levels of IL-8 in normoxic and hypoxic macrophages were below the level of detection (<0.004 IU/ml) at all time points up to and including 4 hours. Macrophages at T_0 (i.e. cells that had been cultured to maturity in 10% serum over 5 days and then lysed immediately prior to normoxic / hypoxic culture in 2% serum) were also found to have undetectable intracellular IL-8. Hence IL-8 release from hypoxic macrophages was not due to release from intracellular stores.

Figure 3.2.7



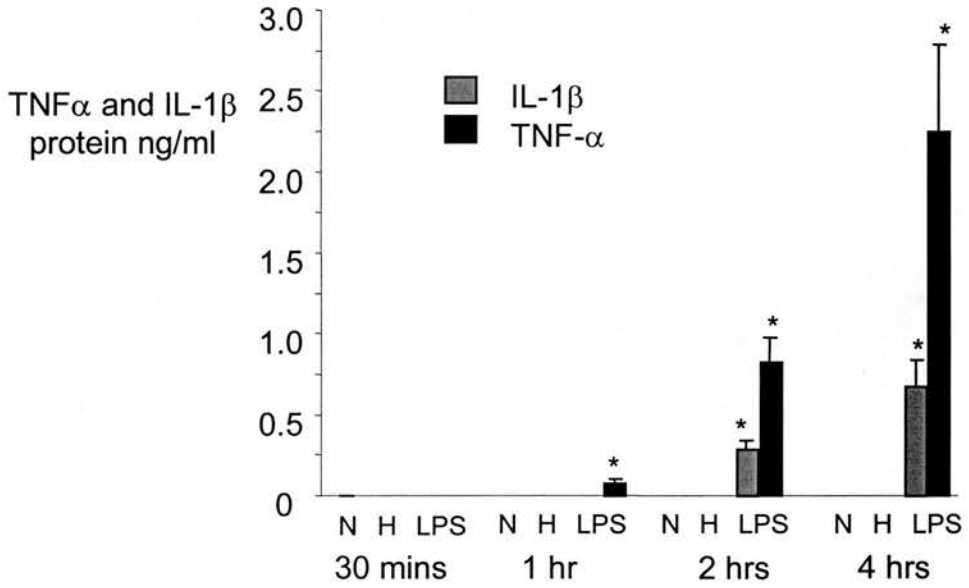
Legend for Figure 3.2.7. IL-8 secretion from human monocyte derived macrophages treated with stress mediators and classical proinflammatory mediators. Macrophages were cultured for 8 hrs (3.2.7**A**) or 2 hrs (3.2.7**B**) with IL-1β (10 ng/ml), TNF-α (10 ng/ml), LPS (1 μg/ml), Substance P (SP, 10^{-10} to 10^{-5} M), adrenalin (Ad, 10^{-10} to 10^{-6} M) or macrophage inhibitory factor (MIF, 0.1-20 ng/ml). At 8 hours, IL-1β, TNF-α, LPS and

Legend for Figure 3.2.7 continued
the higher doses of SP significantly increased IL-8 secretion, and Ad 10^{-6} and 10^{-7} significantly inhibited IL-8 secretion compared to normoxic controls (C). At 2 hours, IL-1β, TNF-α and LPS significantly increased IL-8 secretion compared to controls. Data presented as mean ± S.E.M. n = 5 separate donor experiments for each condition. *P<0.05 compared to controls.

3.2.8. Acute hypoxia does not stimulate macrophage TNF-α or IL-1β secretion.

Secreted levels of both TNF-α and IL-1β were below the level of detection (<15.6 pg/ml and < 3.9 pg/ml respectively) in hypoxic macrophages at all time points (**Figure 3.2.8**). LPS significantly increased secretion of both cytokines by 1 hour and 2 hours respectively. This suggests that the increase in IL-8 secretion by 2 hours hypoxia was phenomenon secondary to increased macrophage TNF-α or IL-1β release.

Figure 3.2.8.



Legend for Figure 3.2.8. Interleukin-1β and TNF-α secretion from hypoxic and LPS treated macrophages. Secreted levels of IL-1β and TNF-α were below the level of detectability from normoxic or hypoxic macrophages. LPS treatment significantly increased secretion of both cytokines compared to normoxic controls. TNF-α secretion following LPS was significantly greater than that of IL-1β at all time points. Data presented as mean ± S.E.M. n=4 separate experiments for each time-point. *P<0.01 compared to normoxic control

3.2.9. The effect of acute reoxygenation and hyperoxia on IL-8 expression in macrophages

The finding that hypoxia increased IL-8 protein secretion by 2 hours was consistent with the premise that acute hypoxia may represent an inflammatory stimulus or 'hit'. However, reoxygenation and hyperoxia potentially represent clinically relevant secondary stimuli or hits. The effects of acute reoxygenation and hyperoxia on macrophage IL-8 generation were therefore studied.

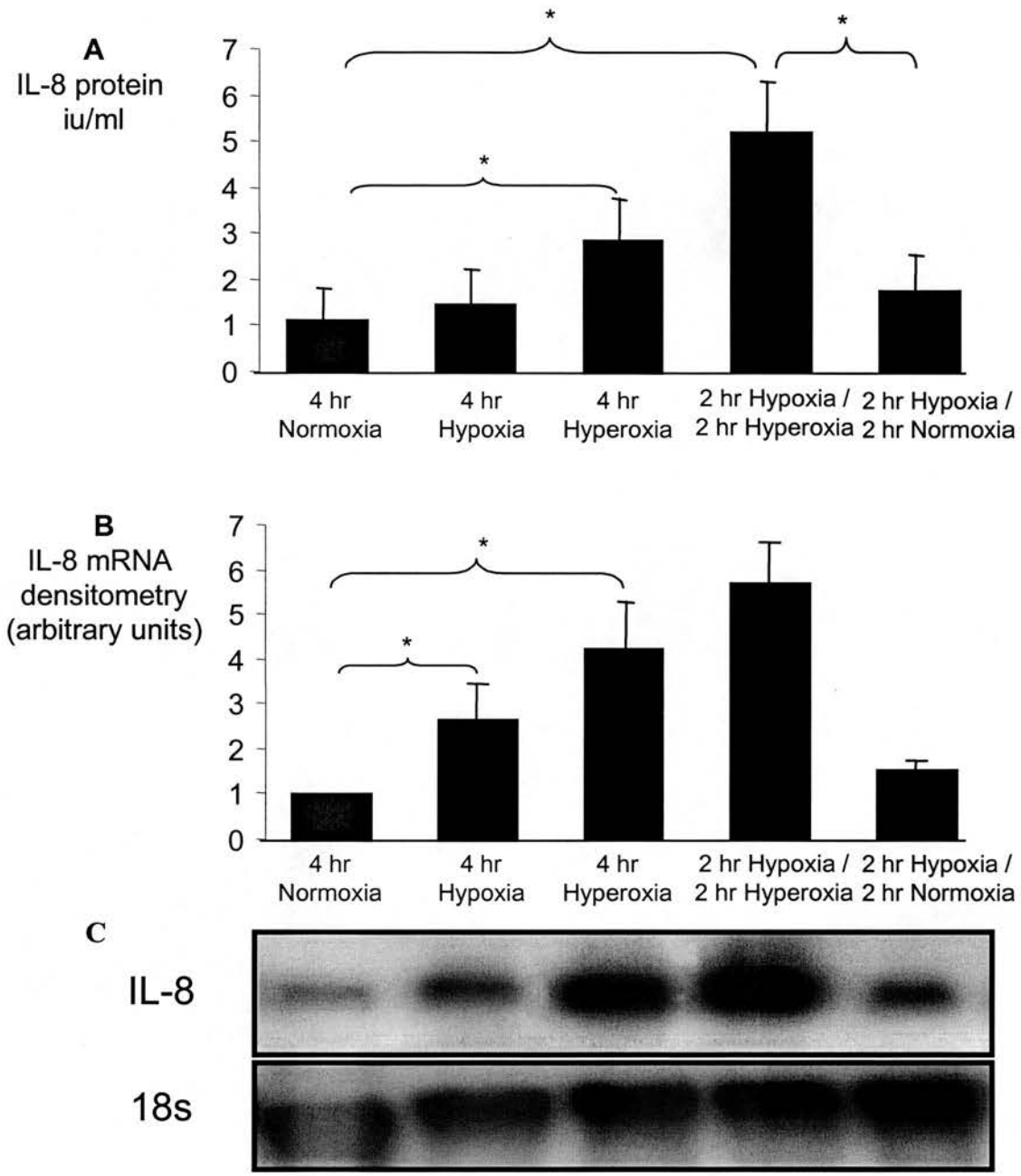
Rapid changes in macrophage oxygenation were achieved by carefully removing hypoxic medium after 2 hours culture, transferring the plates to normoxic (5% CO_2 / 95% air) or hyperoxic (5% CO_2 / 95% O_2) conditions, overlaying cells with fresh medium pre-incubated in the desired atmosphere and culturing for a further 2 hours (as per **Figure 3.2.3.1**). Total IL-8 secreted over the 4 hour experiment was measured by ELISA in pooled supernatants.

Of the conditions tested, hypoxia / hyperoxia was the most potent stimulus for IL-8 generation, resulting in significantly greater protein secretion than hyperoxia / hyperoxia or hypoxia / reoxygenation. Hypoxia / hypoxia did not significantly increase IL-8 protein expression compared to normoxic controls. Steady-state IL-8 mRNA expression paralleled protein expression except for hypoxia / hypoxia, which resulted in a significant increase in mRNA expression without corresponding increase in secreted protein

3.2.10. The effect of acute reoxygenation and hyperoxia on IL-1β and TNF-α expression in macrophages

In contrast to the effects on IL-8 secretion, hyperoxia alone was the most potent stimulus for TNF-α or IL-1β secretion (**Figure 3.2.10**). Reoxygenation to normoxia following hypoxia does not induce detectable levels of either cytokine. In addition, the levels expressed following hyperoxia were <150 pg/ml, approximately ten-fold less than secreted IL-8 levels from macrophages.

Figure 3.2.9.

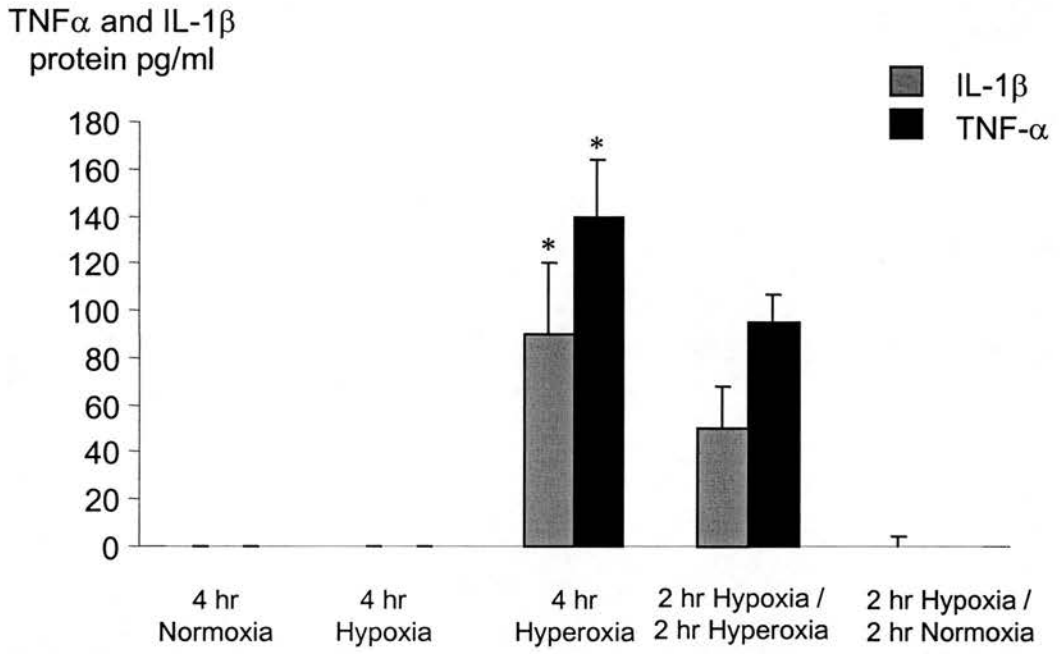


Legend for Figure 3.2.9. The effect of hypoxia, reoxygenation and hyperoxia on IL-8 protein and mRNA expression in macrophages

3.2.9**A.** Hyperoxia resulted in significantly greater IL-8 protein secretion than normoxia or hypoxia. The multiple-hit of hypoxia / hyperoxia was the most potent stimulus for IL-8 protein secretion. Hypoxia / normoxia however resulted in IL-8 levels similar to normoxic controls.

Legend for Figure 3.2.9. continued.
3.2.9**B.** IL-8 mRNA densitometry expressed relative to normoxic control, corrected for 18s (housekeeping gene) expression. As with protein expression, hypoxia / hyperoxia was a more potent stimulus than hyperoxia alone. Hypoxia alone increases IL-8 mRNA expression without corresponding increase in protein secretion. The data for IL-8 protein expression represents mean ± SEM of n=4 separate experiments. IL-8 mRNA expression data represents mean ± SEM from n=3 separate experiments. The northern blot shown (3.2.9**C**) was obtained from RNA derived from macrophages from a single donor.

Figure 3.2.10.



Legend for Figure 3.2.10. The effect of hypoxia, reoxygenation and hyperoxia on IL-1β and TNF-α protein secretion . Hyperoxia significantly increased secretion of both cytokines in monocyte-derived macrophages, and was a more potent stimulus than hypoxia / hyperoxia. The data represents mean ± SEM of n=4 separate experiments. *P<0.05 compared to all other conditions.

3.3. DISCUSSION

In this chapter, the effects of acute hypoxia, reoxygenation and hyperoxia were studied in human monocyte-derived macrophages. The rate of equilibration of culture medium with varying environmental oxygen was shown to occur over several hours, suggesting the need to pre-incubate medium to achieve rapid changes in oxygenation. Acute hypoxia was shown to increase IL-8 protein secretion at 2 hours. The increase at this time-point was comparable to that seen with TNF-α and IL-1β, whilst a selection of stress mediators were shown not to induce IL-8 secretion at this early time-point. Finally, hypoxia / hyperoxia, was found to be a potent stimulus for IL-8 generation. The potential relevance of these findings to raised intrapulmonary IL-8 in trauma patients at-risk of ARDS are discussed.

The inherent limitations of *in vitro* studies in which oxygenation is manipulated have been recognised for many years (Chapman et al., 1970). In order to fully interpret the apparent effects of hypoxia or hyperoxia on cell function, direct measurement of PO_2 in culture medium, ideally in the peri-cellular environment is desirable and perhaps even essential (Ebbesen et al., 2000). In the studies herein, medium (without cells) transferred into hypoxic (0% or 0.1% O_2) or hyperoxic conditions, required up to 5 hours to achieve oxygen equilibration (3.9 KPa and ~70 KPa for hypoxic and hyperoxic environments respectively).

Several factors are likely to influence the rate of equilibration of medium *in vitro*. These include the surface area to volume ratio of the medium, the nature of the plate lid (vented or intact) and convection forces (associated with flushing for example) generated in the incubator. Allen et al., (2001) have demonstrated that in 100-mm plates, lid perforations allowed cell-free medium to equilibrate with a 100% N atmosphere by 2 hours, compared to over 3 hours with intact lids. Equilibration of medium in T-75 flasks with perforated lids, (high surface area : volume) was significantly faster than in 100-mm plates, but still required over 1 hour to achieve. These data support the presented observation that equilibration is not a rapid phenomenon *in vitro*, and justify the use of pre-incubation to achieve rapid changes in oxygenation.

It has been reported that actively metabolising cells significantly affect oxygen equilibrium in hypoxic conditions (Wolff et al., 1993; Metzen et al., 1995; Allen et al., 2001). When medium pre-incubated in anoxic conditions was added to cultured macrophages and maintained in an anoxic environment, the PO_2 fell from ~3.8 KPa to ~3.2 KPa after 4 hours, as measured by OXEL-1 probe. Measurement by automated gas analyser consistently showed a slightly lower value of ~2.8 KPa. It is however unlikely that either method accurately reflected peri-cellular PO_2 in these circumstances. The OXEL-1 probe, which has a tip diameter of 2 mm, was positioned 5 mm above the cell monolayer. The gas-permeable membrane of the OXEL-1 is reportedly affected by protein coating in the presence of cell culture, which may significantly hamper oxygen measurement (Technical Assistance, World Precision Instruments Inc. USA, personal communication). Similarly, the automated gas analyser measures PO_2 in a sample of medium aspirated from the cultured cells, and is not a direct measure of peri-cellular oxygenation. Both methods are therefore likely to have underestimated the degree of hypoxia experienced by cells in this environment. In the presence of constant oxygen utilisation in an anoxic environment, it is likely that the peri-cellular PO_2 would be lower.

Using a fibre-optic oxygen probe (which is not affected by protein fouling), positioned 1.5 mm above confluent A549 cells, Allen et al., (2001) showed that the peri-cellular PO_2 fell from ~20 KPa to 0 KPa by 90 min of transfer to an anoxic environment. Previous studies in which PO_2 in cell cultures has been directly measured or estimated have also suggested very low oxygen tensions in the peri-cellular region (McLimans et al., 1968; Stevens, 1965). Indeed, it has been shown that even in *normoxic* conditions (21 % oxygen), the measured peri-cellular PO_2 of hepatoma (HepG2 and Hep3B) cells is zero, compared to ~10 KPa in a bovine endothelial cell-line and 15 KPa in renal epithelial cells (Metzen et al., 1995). These data expose a potentially critical aspect of oxygenation studies; the standard classification employed in studies of hypoxia, normoxia and hyperoxia do not reflect physiological cellular oxygenation *in vivo*, and neither do they necessarily reflect cellular oxygenation *in vitro* culture. Hence, 'normoxia' in *in vitro* studies is generally accepted as equivalent to ambient air, though this is only likely to be biologically relevant to the lining cells of the upper respiratory tract (**Figure 1.4.1**).

Exposing hepatoma cells to severe hypoxia *in vitro* would seem a reasonable model for studying the pathology of hepatic ischaemic injury, but the observation that the peri-cellular PO_2 of 'normoxic' hepatoma cells approaches zero needs consideration when interpreting the results. In the light of these observations, it has recently been proposed that studies of oxygenation *in vitro* should attempt to directly measure peri-cellular oxygenation and to define normoxia, hypoxia and hyperoxia based upon current knowledge of physiologically relevant PO_2 for individual cell-types cell in health and in disease states (Ebbesen et al., 2000).

Interleukin-8 protein secretion from macrophages under hypoxic conditions was significantly higher than normoxic controls by 2 hours, but not at earlier time-points. By 4 hours, protein secretion from hypoxic cells was similar to controls. It is apparent that even under 'basal' normoxic controls, macrophages continue to secrete IL-8. It is possible that the switch from 10% serum in which the macrophages were derived, to 2% serum for experiments, acted as a 'serum-withdrawl' stimulus. It is also possible that the 2 hour effect is a response to a progressively falling pericellular PO_2 as equilibrated hypoxic medium is added to actively metabolising cells. An inter-donor variation in the 2 hour hypoxic response was observed which may reflect an underlying genetic polymorphism, several of which have recently been described for the IL-8 gene (Hull et al., 2001; Renzoni et al., 2000; Emi et al., 1999). However, it should be emphasised that as all the experiments were performed in the presence of autologous serum, the hypoxic IL-8 response may reflect the presence of an unidentified serum factor or factors.

The magnitude of the IL-8 response by 2 hours hypoxia was comparatively smaller than that observed with LPS, it was similar to that seen with TNF-α and IL-1β stimulation. This comparison is valid since endotoxin, TNF-α and IL-1β are well-characterised inflammatory mediators with established roles in the pathogenesis of the inflammatory response (Morrisson and Ryan, 1987; Tracey et al., 1986; Tracey and Cerami, 1994; Dinarello, 1992). Elevated levels of both cytokines are found in the alveolar airspaces of patients both at-risk of, and with, established ARDS (**Chapter 1.1**). The clinical relevance of endotoxin in trauma patients, in whom there is no obvious cause for endotoxaemia, is unresolved. The translocation of endotoxin from bowel under conditions

of hypo-perfusion and hypotension has been demonstrated in animal models and patients in an intensive care setting (Moore et al., 1991; Rouman et al., 1993C; Rush et al., 1988). However, the association between gut translocation and the development of MODS or ARDS remains contentious (Hoch et al., 1993; Ayala et al., 1990; Donnelley et al., 1994A; Saadia and Lipman, 1995; Hassoun et al., 2001). In our own study of trauma patients, we found no association between initial endotoxin levels in BAL fluid or blood and progression to ARDS (Hirani et al., 2001).

In contrast to the much-studied roles of endotoxin, TNF-α and IL-1β in trauma and the inflammatory response, there is comparatively little *in vitro* or *in vivo* data regarding stress mediators in this setting. Substance P (SP), adrenalin and MIF may however be relevant to the acute systemic inflammatory process, and in particular to the at-risk period of ARDS. In human studies, raised plasma SP levels have been reported following soft-tissue injury and burns when compared to healthy controls (Onuoha and Alpar, 1999; Onuoha and Alpar, 2001). The plasma concentration of SP in injured patients, measured within 24 hours of injury, was in the picomolar range. Within the lung, SP secreting fibres are present at all levels of the airways, as part of the non-adrenergic, non-cholinergic (NANC) neural network (Fox et al., 1980; Barnes, 1986). In a study of seven patients with established ARDS, BAL levels of SP were in the 10^{-10} range and 3-4 fold higher than in controls with congestive heart failure (Espiritu et al., 1992). The potential importance of SP in acute lung injury has been demonstrated in studies of genetically engineered mice deficient in the SP receptor NK-1R. In wild-type mice, intravenous delivery of chicken egg albumin and intra-tracheal rabbit antibody to chicken egg albumin resulted in an acute neutrophil influx and alveolar protein leak. Lung injury was significantly attenuated in NK-1R knockout mice (Bozic et al., 1996). A similar observation was made in a pancreatitis-induced model of ALI (Bhatia et al., 1998 in PNASU). Finally, a specific NK-1R antagonist has been shown to attenuate immune complex mediated lung injury in mice (Kaltreider et al., 1997).

In the presented study, SP was found to have no effect on human macrophage IL-8 release by 2 hours. However at doses of 10^{-7} to 10^{-5} M, SP significantly increased IL-8 secretion by at 8 hours. In previous studies, human monocytes have been shown to

secrete IL-1, TNF-α and IL-6 in response to SP by 6 hours, at an optimal dose of 10^{-8} M (Lotz et al., 1988). SP at doses in the nanomolar range increases TNF-α secretion from human cord blood monocyte-derived macrophages (Ho et al., 1996), and IL-8 secretion from human astrocytoma cells (Lieb et al., 1997) over 24 hrs. Hence, SP represents a pro-inflammatory mediator, which may be relevant in the pathogenesis of acute lung injury and ARDS. However, it was found not to be a rapid inducer of IL-8 generation from macrophages *in vitro*.

Following a major insult, the stress-induced 'fight or fight' reaction is principally mediated through enhanced production of catecholamines. Reported maximum plasma adrenalin levels in unstressed, supine, normotensive control human subjects are 100-150 pg/ml (Cryer, 1980; Rosano et al., 1991). Immediately following trauma for example, levels increase 10 to 100–fold, with a near linear relationship between severity of injury and plasma adrenalin (Little et al., 1985). Adrenoceptors have been identified on pulmonary endothelial, epithelial, smooth muscle and macrophage cells (Barnes, 1984). Adrenergic innervation in the human lung is however comparatively sparse (Barnes, 1986) and this suggests that the majority of intrapulmonary effects are mediated by circulating catecholamines. In the presence of lung leak, a feature of acute lung injury, alveolar cells will be exposed to plasma adrenalin. In patients with established ARDS, circulating adrenalin levels measured at a single time-point have been reported to range from 0 to 75,000 pg/ml (Ware and Matthay, 2001).

In monocyte-derived macrophages, adrenalin inhibited basal IL-8 secretion by 8 hours, but had no effect at 2 hours. In general, adrenalin is considered to promote anti-inflammatory responses (Uusaro and Russell 2000), including inhibiting TNF-α production (Liao et al., 1995; Monastra and Secchi, 1993; van der Poll et al., 1996). In LPS-stimulated THP-1 cells, (a human macrophage cell line) and human whole blood, adrenalin significantly inhibited TNF-α production over 7 hours (Severn et al., 1992). However, in contrast to the inhibitory effects on TNF-α, there is evidence that catecholamines may enhance IL-8 production. Adrenalin (10^{-7} to 10^{-5} M) increased IL-8 production in whole blood co-treated with LPS over 16 hours (van der Poll and Lowry, 1997). Similarly, isoprenalin (10^{-7}), a potent β_2-receptor agonist was found to increase

basal IL-8 secretion from a bronchial epithelial cell line over 18 hours (Linden, 1996). It is likely therefore that the cytokine response to adrenalin is cell-specific.

Macrophage migration inhibitory factor (MIF) has been identified as a potentially important proinflammatory stress mediator (Bucala, 1996; Donnelly et al., 2000). Median circulating levels of MIF in multiple-trauma patients are reportedly ~ 20 ng/ml, fourteen-fold higher than controls (Joshi et al., 2000). Within the lung, raised MIF levels in the nanogram range in patients with established ARDS (Donnelly et al., 1996) and asthma (Rossi et al., 1998) have been reported. Whilst some intrapulmonary MIF is likely derived from plasma in patients with lung leak, the alveolar macrophage is also identified as a source of IL-8 (Donnelly et al., 1996). At doses of 0.1 – 20 ng/ml, MIF did not stimulate IL-8 generation from macrophages at 2 or 8 hours. In contrast, Donnelly et al., (1996) have previously shown that MIF 10 ng/ml increased IL-8 generation fromalveolar cells lavaged from patients with ARDS.

There are clearly a number of important differences between cells from ARDS lavage samples and monocyte-derived macrophages, which could account for the different IL-8 responses seen with MIF. Firstly, alveolar macrophages typically represent only 30-60 % of lavage cells from ARDS lungs, the remainder being neutrophils (Holter et al., 1986; Steinberg et al., 1994; McGuire et al., 1982). The relative contribution to MIF generation from these two cell populations is not known. Secondly, the monocyte-derived macrophage, relative to the alveolar macrophage, may be MIF insensitive. Lastly, cells derived from patients with ARDS may represent an 'activated' phenotype, in contrast to the comparatively 'quiescent' monocyte-derived macrophages studied in the current experiments.

Having established that 2 hours of hypoxia upregulated IL-8 protein secretion, the effects rapid reoxygenation and hyperoxia were studied. Acute hyperoxia following acute hypoxia was found to be a greater stimulus for IL-8 generation than hypoxia, hyperoxia or hypoxia / normoxia. Cell viability, as measured by trypan blue exclusion and LDH release, was found to be unaffected by hypoxia, reoxygenation or hyperoxia over the time-periods studied. In general, it appears that macrophages are reasonably tolerant of

oxygen-deprivation (Lewis et al., 1999). Human monocyte-derived macrophages were found not only to be viable for up to 48 hours in a 2% oxygen atmosphere, but also to increase expression of ORP150 (oxygen-regulated protein 150 kDa), a novel protein which may convey protection from apoptosis (Tsukamoto et al., 1996). Guinea pig alveolar macrophages retain high viability even after 24 hours in an anoxic environment (Cazin et al., 1990). In Kupffer cells, reoxygenation following a period of hypoxia appears to inflict greater injury than hypoxia alone, though significant necrosis only occurs after several hours of experimental exposure (Rymsa et al., 1990; Rymsa et al., 1991). Alveolar macrophages remain viable for at least 24 hours in hyperoxic (85 - 95% O_2) culture (Deaton PR et al., 1994; Desmarquest et al., 1998; Pepperl et al., 2001).

Several studies have reported the effects of *prolonged* hypoxia and or hyperoxia on cytokine generation in macrophages *in vitro* (O'Brien-Ladner et al., 1995; Deaton et al., 1994; VanOtteren et al., 1995). In human monocytes, anoxia / hyperoxia over a 24 hour period was found to be a potent stimulus for IL-8 generation (Metinko et al., 1992). Alveolar macrophages from patients with interstitial lung disease, secreted increased IL-8 protein in response to 24-48 hours hyperoxia whilst inhibiting TNF-α, IL-1β and IL-6 release (Desmarquest et al., 1998).

In contrast to these studies of prolonged exposure, there have been relatively few studies exploring the acute effects of hypoxia and reoxygenation in macrophages *in vitro*. Koga et al., (1992) demonstrated that 3 hours hypoxia (<4 KPa as measured in atmosphere, without pre-incubation of medium) followed by 3 hours normoxia was insufficient to generate IL-1 from human monocyte-derived macrophages, but that after 6 hours reoxygenation, increased levels of IL-1 were detected in supernatants. Even prolonged exposures however failed to induce detectable TNF-α secretion. Interpretation of the effects of acute exposure to changes in oxygenation is dependent upon the precise methodology. In studies in which medium or peri-cellular PO_2 is not directly measured, the effects or lack of effects may reflect time taken for the medium to equilibrate.

Finally, it is appropriate to consider if the levels of hypoxia and hyperoxia in the *in vitro* studies are clinically relevant in the airspaces of trauma patients at-risk of ARDS. In health, the mean alveolar PO_2 is ~14 KPa, and this is therefore likely to represent the average level of oxygenation to which the alveolar macrophage would be exposed. Following major trauma, hypoventilation, atelectasis, pulmonary contusion and aspiration are all likely to contribute to regional alveolar hypoxia (Julien et al., 1987; Silverston, 1989; Vlessis and Trunkey, 1997; Mizushima et al., 2000; Miller et al., 2001). Direct measurement of alveolar PO_2 in this acute setting is not feasible. Parameters such as PaO_2 / FiO_2, afford a measure of overall gas exchange but do not reflect alveolar PO_2. The observation that virtually all our studied trauma victims exhibited impaired gas exchange (PaO_2 / FiO_2 median 42 KPa, range 27 – 61) in the absence of radiographic evidence of ARDS, suggests the presence of shunting, presumably through atelectatic and contused lungs (Hirani et al., 2001). However, the correlation between reduced PaO_2 / FiO_2 and IL-8 was only moderately strong (r= - 0.56). Miller et al., (2001) have shown a strong association between the extent of contusion injury and the development of ARDS. In spite of these supporting data, it remains that there is no direct evidence to show that the alveolar macrophage is subject to severe hypoxia in the trauma setting.

In contrast to hypoxia, the concept that alveolar macrophages are exposed to hyperoxia is entirely plausible in trauma patients during resuscitation. The delivery of high flow oxygen (60-100%) is considered mandatory in severely injured patients (Trunkey, 1991; American College of Surgeons Committee on Trauma, 1997). In a ventilated patient, alveolar PO_2 levels of 60-90 KPa would be expected under these circumstances. Furthermore, if the patient is intubated and receives positive-pressure ventilation, as in our reported study, then previously atelectatic (and presumed hypoxic) regions of the lung will be recruited and exposed to hyperoxia. This forms the basis of acute hypoxia / hyperoxia as potential multiple hits patients at-risk of ARDS, and is further explored in an *in vivo* model of lung injury.

CHAPTER 4

HYPOXIA / HYPEROXIA IN AN *IN VIVO* MODEL OF ACUTE LUNG INJURY

4.1 INTRODUCTION

The multiple-hit hypothesis has been previously advocated as a model to account for the evolution of the multiple-organ dysfunction syndrome (MODS) following a major insult such as trauma or sepsis (Moore and Moore, 1995; Bone, 1996; Saadia and Lipman, 1996). It may however be equally applicable to the development of ARDS. In this model, it is postulated that the initial systemic inflammatory response is appropriate to the primary clinical event or hit and is aimed at maintaining or upregulating the host immune system and initiating wound healing. However a second hit (or hits) generates an exaggerated or overwhelming inflammatory response, beyond the requirement for maintaining host defence and precipitating tissue injury (**Figure 4.1.1**). This model of evolving inflammation seems intuitively attractive, in part since it accommodates the chronological progression of events in patients who progress to MODS and ARDS.

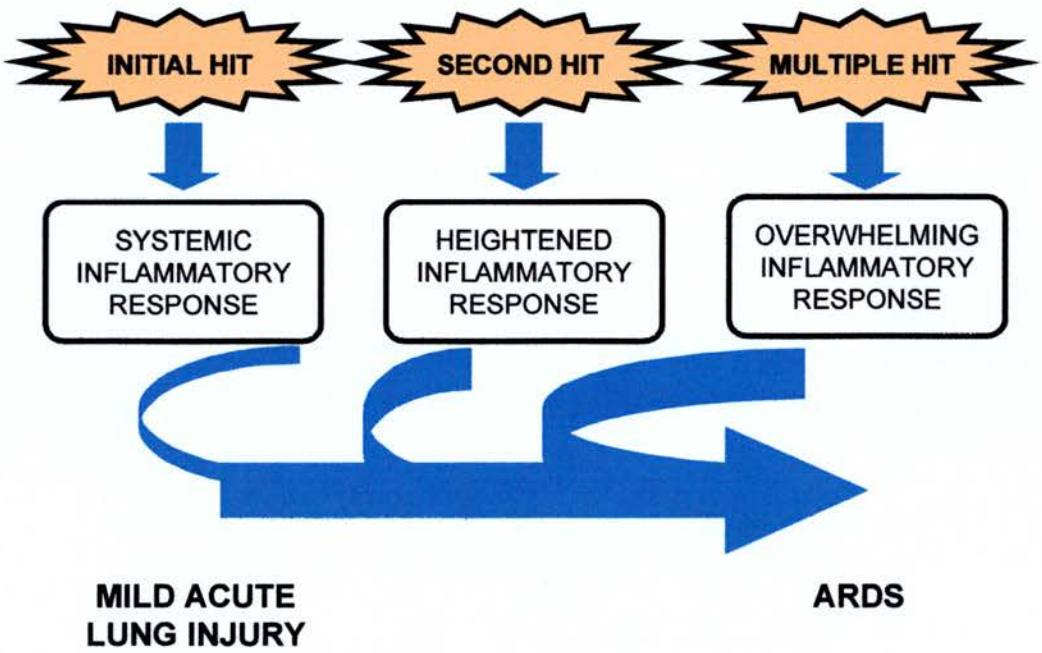
For the purpose of this study, it was postulated that multiple-hits, occurring sequentially and / or simultaneously, may be responsible for rapid IL-8 generation in the lungs of patients that progress to ARDS. The precise nature of these hits is speculative. In Chapter 3, acute hypoxia and hyperoxia were proposed as clinically relevant potential hits, and were found to upregulate IL-8 synthesis in human monocyte-derived macrophages. However, in the clinical practice, hypoxia and hyperoxia do not generally occur in isolation, but rather in the context of a systemic inflammatory response initiated by a significant insult. The potential role of oxygenation in this complex setting may be better studied in an *in vivo* model

Several animal models of acute lung injury have been described and have greatly advanced our understanding of the pathogenesis of ARDS (Vedder et al., 1988; Sekido et al., 1993; Folkesson et al., 1995; Czermak et al., 1999; Frevert et al., 2000; Nagase et al., 2000). Since our recent clinical data reported raised intrapulmonary IL-8 levels specifically in trauma patients (Hirani et al., 2001), it would seem appropriate to complement these studies with a trauma model in which to test the multi-hit hypothesis. A number of such models have been described (Armstead et al., 1997; Rawlins et al., 1999; Rubinstein et al., 1998; Bless et al., 1999), but these may be subject to both

logistical and ethical constraints. Furthermore, our observations suggest that the relationship between raised alveolar IL-8 and progression to ARDS is not peculiar to trauma patients, but is valid in a variety of conditions associated with ARDS (Donnelly et al., 1993).

A novel animal model of localised acid instillation in an anaesthetised, ventilated rabbit was developed. The methodology is described in Chapter 2. The principle aim was to generate a reproducible model of acute lung injury, in which to test the specific hypothesis that acute hypoxia / hyperoxia, was a stimulus for rapid intrapulmonary IL-8 generation *in vivo*.

Figure 4.1.1



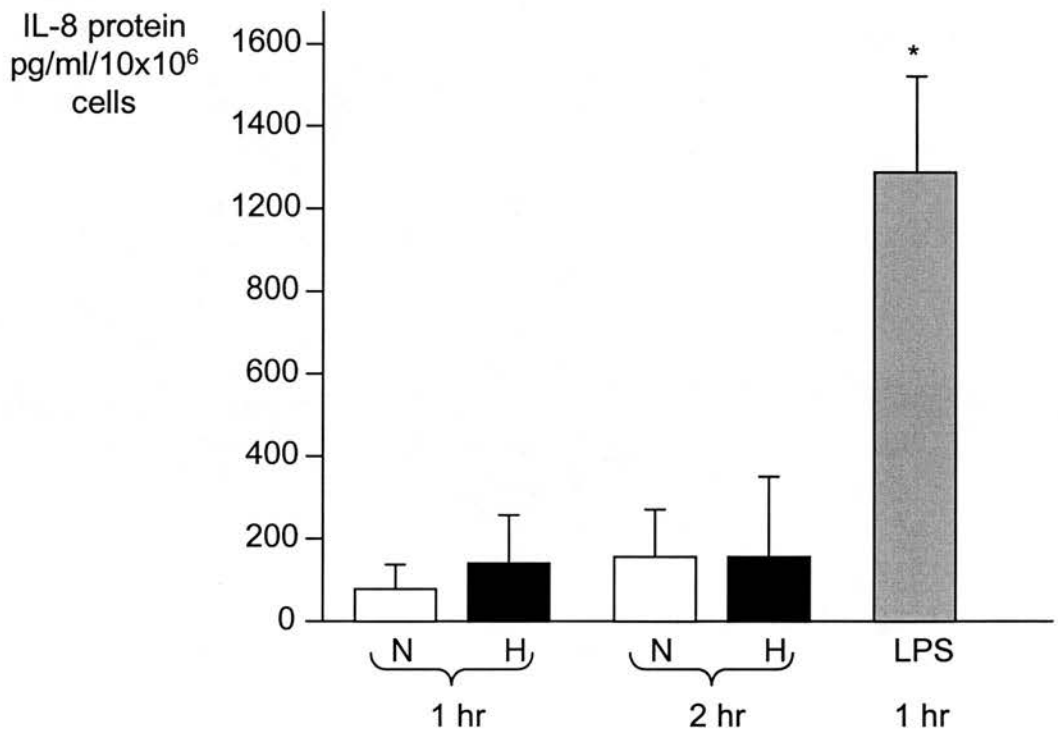
Legend for Figure 4.1.1. A multi-hit model for the pathogenesis of ARDS.
The precise nature of the hits remains speculative. Acute hypoxia and hyperoxia may however represent clinically relevant multiple hits, which through increasing intrapulmaonary IL-8 generation drive the inflammatory process from mild lung injury to ARDS.

4.2 RESULTS

4.2.1. and 4.2.2 Acute hypoxia increases IL-8 mRNA but not IL-8 protein expression in rabbit alveolar macrophages

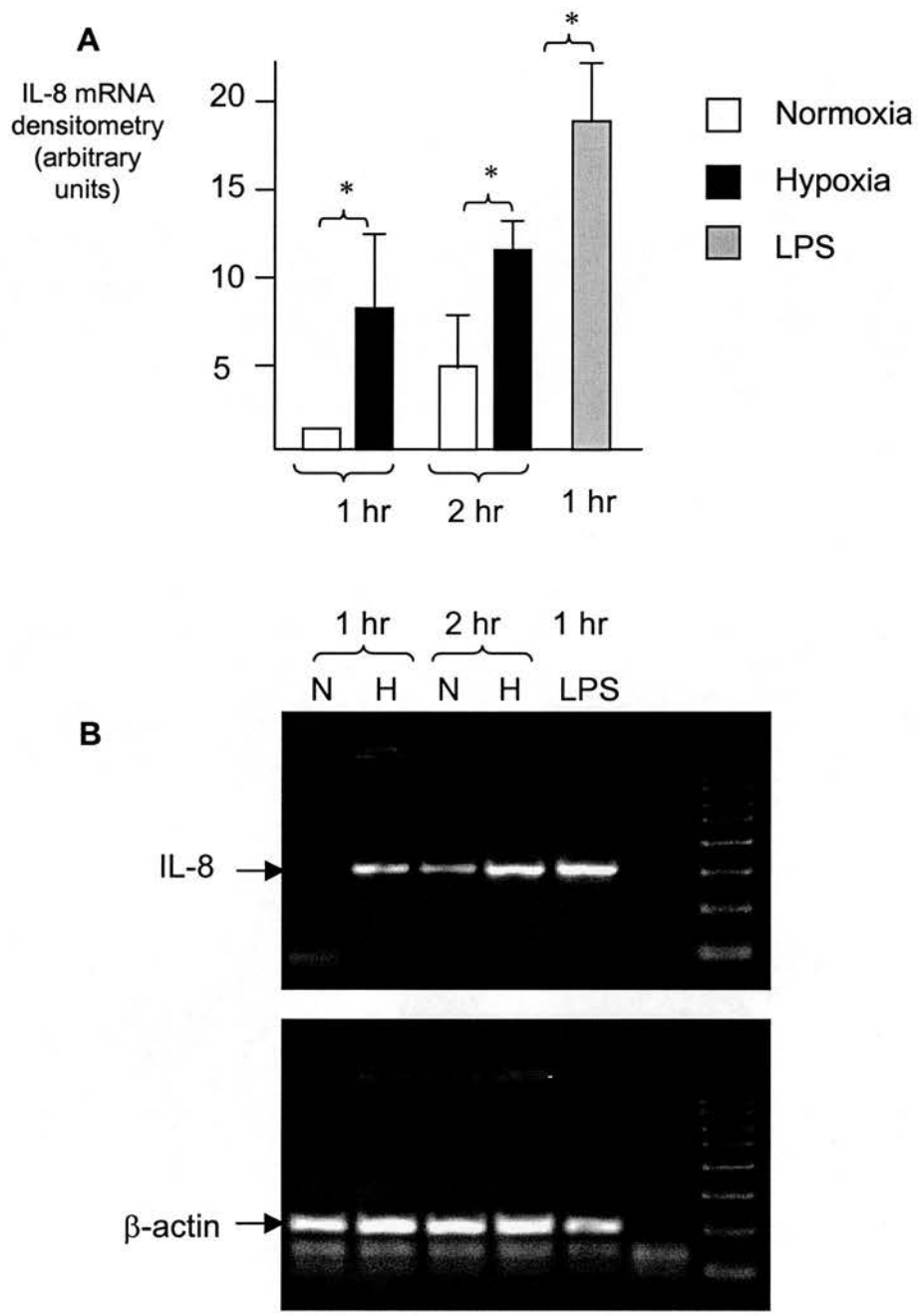
In chapter 3, two hours hypoxia was found to be a stimulus for IL-8 protein generation in human monocyte-derived macrophages. To establish if rabbit alveolar macrophages responded similarly to hypoxia, cells lavaged *ex-vivo* from healthy rabbits were exposed to hypoxia for up to 2 hours. IL-8 protein secretion from hypoxic alveolar macrophages was not significantly different to that from normoxic cells (**Figure 4.2.1**). Steady-state IL-8 mRNA expression, as measured by RT-PCR, was however significantly higher in hypoxic cells (**Figure 4.2.2**)

Figure 4.2.1



Legend for Figure 4.2.1. The effect of acute hypoxia on rabbit alveolar macrophage IL-8 protein secretion.
Alveolar macrophages cultured in 10% FCS were incubated in hypoxic conditions for up to 2 hours. IL-8 protein secretion was not significantly different to normoxic controls. LPS (1 ng /ml) significantly increased IL-8 release by 1 hour. The data is presented as mean ± SEM of n=3 separate experiments performed in triplicate. *P<0.02 compared to normoxic controls.

Figure 4.2.2



Legend for Figure 4.2.2. Effect of acute hypoxia on rabbit alveolar macrophage IL-8 mRNA expression.

Alveolar macrophages cultured in 10% FCS were exposed to acute hypoxia for up to 2 hours. In contrast to IL-8 protein secretion, IL-8 mRNA expression was significantly higher than normoxic controls. LPS (1 ng/ml) significantly increased IL-8 m RNA expression by 1 hour. Each value in 4.2.2**A** represents mean ± SEM of n=3 separate experiments. *P<0.05 compared to normoxic controls. A representative RT-PCR gel is shown in 4.2.2**B.**

4.2.3 Localised acid instillation induces direct lung injury in the instilled lobe but no injury in the contralateral lung, in normoxic ventilated rabbits

In anaesthetised, ventilated rabbits, HCl was carefully instilled into the left lower lobe of lung under direct vision, via a bronchoscope. Control animals were instilled with an equal volume of air. The animals were then ventilated under normoxic conditions (FiO_2 ~20%, arterial PO_2 ~10.5 KPa) for up to 4 hours. Ventilation strategy was identical in control and acid-instilled animals, and there was no significant difference in arterial PO_2, PCO_2 and pH, heart-rate and blood pressure between control and acid-instilled animals (**Table 4.2.1**). Both right and left lower lobes were then lavaged. The degree of lung injury was assessed by the neutrophil count and alveolar-capillary (lung) protein leak as measured by detection of Evans Blue / BSA in BAL fluid. In some animals, BAL was not performed to allow histological assessment of lung injury to be made without lavage artefact.

The lung injury data for directly-instilled lungs and contralateral lungs at 2 and 4 hours post instillation in animals ventilated under normoxic conditions is summarised in **Table 4.2.2**. By 2 hours, compared to saline-instilled controls, acid-instillation induced lung injury, as quantified by a 14-fold increase in lung leak and significantly raised BAL neutrophils in the directly injured lobe. The degree of lung injury as measured by these parameters was not significantly different by 4 hours. In contrast, localised acid instillation did not cause significant lung injury in the contralateral lung by 4 hours. Representative BAL cytospins from control and acid injured lungs at 2 and 4 hours are shown in **Figure 4.2.3.1** Histology of acid-injured lung at 2 and 4 hours post-instillation revealed neutrophil influx into the lung interstitium and airspaces. There was evidence of proteinaceous leak with fibrinoid deposition in the alveoli. The majority of the acute inflammatory infiltrate was located centrally in the lung. Histology of the contralateral lungs showed no evidence of lung injury at 2 or 4 hours (**Figure 4.2.3.2**)

Table 4.2.1

	EXPERIMENTAL GROUP						
	CONTROL (NO ACID)		LOCALISED ACID INSTILLATION				
	2 Hr Normoxia n=3	4 Hr Normoxia n=3	2 Hr Normoxia n=4	4 Hr Normoxia n=6	2 Hr Hypoxia n=4	2 Hr Hypoxia / 2Hr Hyperoxia n=6	2 Hr Hypoxia / 2 Hr Normoxia (Controlled reoxygenation) n=4
Animal weight (Kg)	3.3±0.2	3.2±0.3	3.0±0.5	3.1±0.2	3.1±0.4	3.0±0.3	2.9±0.3
Respiratory Rate (breaths / min)	38±2	38.8±1.5	36±3.5	36.3±1.5	34.6±2.8	35.8±3.1	34.0±3.2
Peak Inspiratory Pressure (cm H_20)	12.6±0.8	12.9±0.6	12.5±1.0	12.5±0.6	11.8±1.0	12.1±0.9	12.3±0.8
FiO2 (%)	21	21	21	21	17	17 / 100	30
Heart rate (beats per min)	220±14	211±21	218±14	201±16	222±18	261±10*	216±14
Mean systemic arterial pressure (mmHg)	64±6	60.5±5.3	68±4.9	62.3±5.7	63.5±6.2	68±3.7	62.5±6
Arterial pH	7.4±.05	7.43±.06	7.45±.06	7.43±.07	7.40±.10	7.43±0.02	7.39±0.08
Arterial PCO_2 (KPa)	4.7±0.7	4.3±0.31	4.7±0.51	4.1±0.37	33±1.5	4.7±0.5	4.3±0.42
Arterial PO_2 (KPa)	11.2±0.5	11.4±0.6	11.4±0.5	11.2±0.4	5.1±0.22	5.3±0.2 / 64±4	5.1±0.2 / 15±1.3

Table 4.2.1. Ventilatory and physiological parameters in experimental animals
The required variation in arterial PO_2 in experimental groups was achieved through altering FiO_2 only. Localised instillation of acid into the left lower lobe did not have any significant effect on heart rate, mean arterial pressure, pH, PO_2 or PCO_2 compared to control animals over 2 or 4 hours. Hypoxic ventilation with a FiO_2 of 17% achieved a target arterial PO_2 of ~5 KPa. However, significant hypotension and acidosis occurred after approximately 3 hours of hypoxic ventilation (data not shown). Hence, hypoxia experiments were performed for up to 2 hours only with equivalent normoxic controls. Ventilatory parameters were not significantly different between experimental groups. Other than a significant increase in heart rate in the hypoxic / hyperoxic group, there was no significant difference in physiological parameters between groups. Data presented as mean ± SEM, except heart rate (median ± SD). *P=0.04

Table 4.2.2

	2 Hours			4 Hours		
	Control n=3	Acid Instillation n=4		Control n=3	Acid Instillation n=6	
		Contralateral lung	Directly injured lung		Contralateral lung	Directly injured lung
Lung protein leak (ml)	0.028±0.01	0.02±0.01	0.39±0.1*	0.033±0.02	0.038±0.01	0.44±0.08**
BAL neutrophils (%)	0.8±0.5	1.0±0.4	34±14*	1.1±0.5	1.2±0.8	35±10**
BAL neutrophils cells x 10^5 / 10 ml BAL fluid	0.12±0.07	0.18±0.06	23.2±8*	0.14±0.12	0.18±0.4	22.5±4**

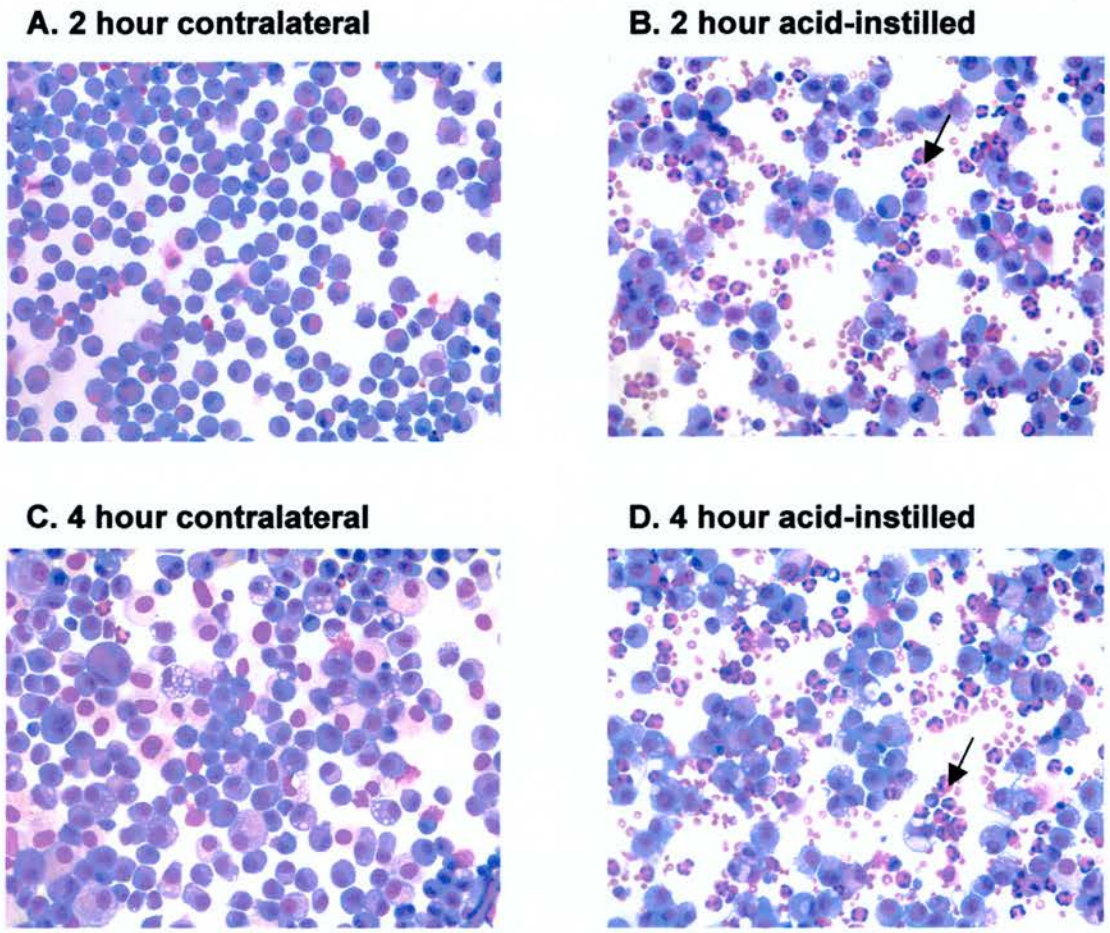
Table 4.2.2. Localised acid instillation on measures of lung injury at 2 and 4 hours in animals ventilated under normoxic conditions.
Data are presented as means± SEM, except BAL neutrophils, which are represented by median ± SD. *P < 0.01 compared to 2 hour control animals and 2 hour contralateral lung. **P < 0.01 compared to 4 hour control animals and 4 hour contralateral lung.

4.2.4. Localised acid instillation increases IL-8 protein expression in the directly injured but not the contralateral lung

Compared to controls (no acid), acid-injured lungs expressed significantly greater IL-8 protein in BAL fluid (162±19 v 90±12 pg/ml, P<0.02) (**Figure 4.2.4.1**) and lung homogenate (635±68 v 312±85 pg/ml/mg P<0.02) (**Figure 4.2.4.2**) by 2 hours. In the contralateral lung, compared to control animals, neither BAL IL-8 levels nor lung homogenate IL-8 levels were significantly different. The values at 4 hours were similar, with significantly raised IL-8 in the acid-injured lungs and levels similar to control animals in the contralateral lung.

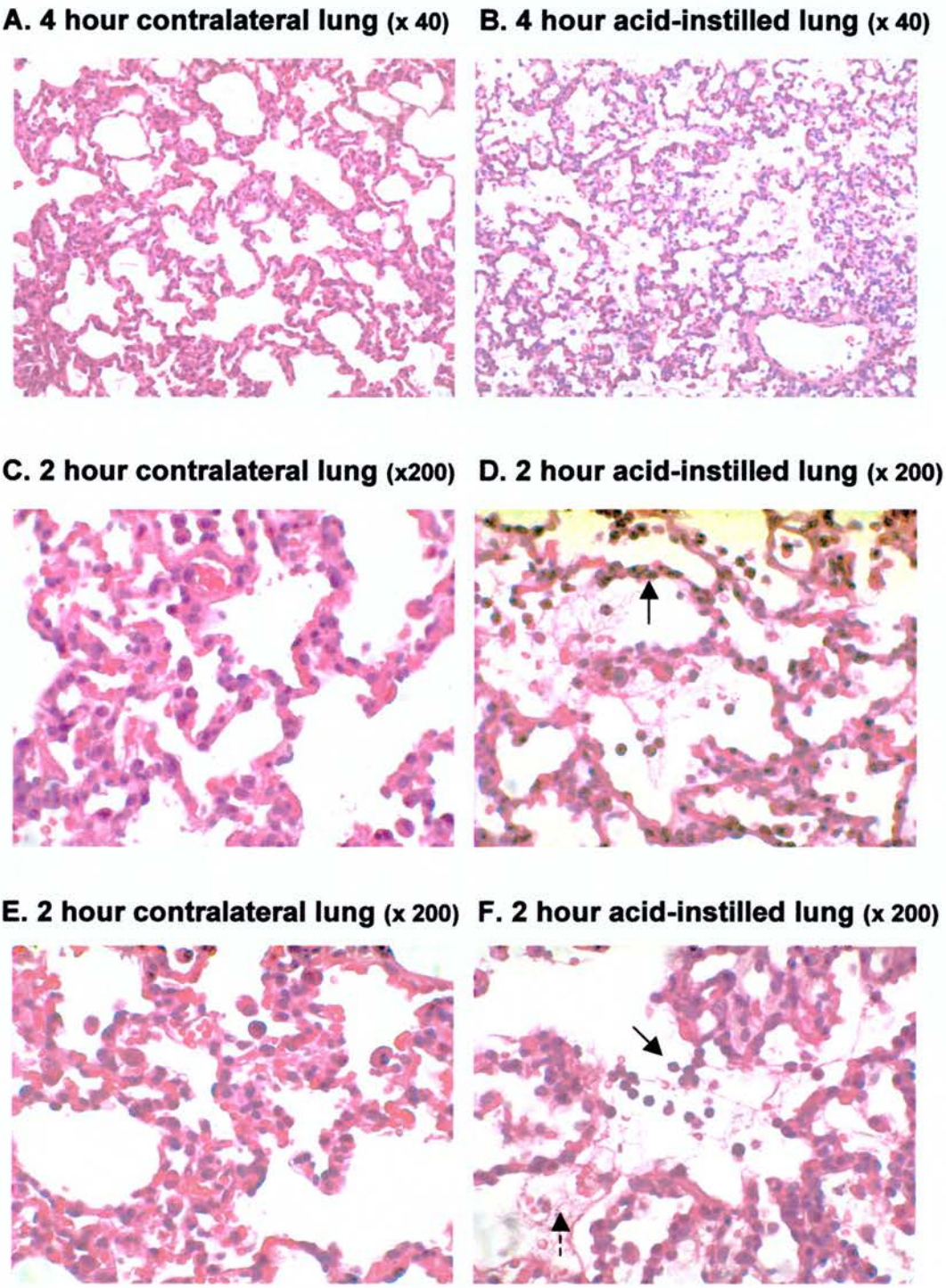
Serum levels of IL-8 were not detectable at any time point during experiments in acid-instilled or control animals.

Figure 4.2.3.1



Legend for Figure 4.2.3.1. Bronchoalveolar lavage cytospins from acid-injured and contralateral lungs.
Rabbits were ventilated for 2 hours and 4 hours under normoxic conditions following localised acid instillation into the left lower lobe. Both directly-injured and contralateral lungs were lavaged. The cytospins show presence of neutrophils (arrowed) in the directly-injured, but not contralateral lungs. (Stained with DiffQuick, modified Wright-Giemsa)

Figure 4.2.3.2



Legend for Figure 4.2.3.2. Histology of acid-injured and contralateral lungs.
Rabbits were ventilated for 2 hours and 4 hours under normoxic conditions following localised acid instillation into the left lower lobe. Tissue sections were taken from non-lavaged lungs. Acid-instillation resulted in neutrophil infiltration into the lung interstitium and airspaces (solid arrows). Presence of fibrin (broken arrow) in the alveolar space indicates proteinaceous fluid leak.

Having established a reproducible model of direct and potentially indirect lung injury, the effects of acute hypoxia and hyperoxia on intrapulmonary IL-8 generation were studied. As described in **Chapter 2.2**, changes in arterial oxygenation were affected through reducing or increasing FiO_2 only, without altering ventilatory parameters. **Table 4.2.1** summarises the levels of oxygenation achieved, and shows that the ventilation strategy between experimental groups was similar. However, prolonged hypoxia beyond 3 hours resulted in significant reduction in arterial pH (data not shown), hence hypoxia experiments were limited to 2 hours. Varying oxygenation had no significant effect on physiological parameters other than heart-rate, which was significantly higher in the hypoxia / hyperoxia group.

4.2.4. Acute hypoxia has no effect on acute lung injury

Following acid instillation, animals were ventilated under hypoxic (FiO_2 17%, arterial PO_2 ~5 KPa) or normoxic conditions.

Hypoxia for 2 hours had no significant effect on, protein leak or BAL neutrophils in either the directly injured or contralateral lung (**Table 4.2.3**).

Table 4.2.3

	Contralateral lung		Directly-injured lung	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Lung protein leak (ml)	0.02 ± 0.01	0.032 ± 0.02	0.39 ± 0.1	0.32 ± 0.06
BAL PMN (%)	1.0 ± 0.4	1.3 ± 0.4	34 ± 14	30 ± 8
BAL PMN (cells x10^5 / 10 ml BAL fluid)	0.18 ± 0.06	0.22 ± 0.06	23.2 ± 8	21.6 ± 9

Legend for Table 4.2.3. Effect of 2 hours hypoxia on lung injury in acid-induced lung injury

Results are presented as mean ± SEM, except BAL PMN (%) which are represented by median ± SD. There is no significant difference in measures of lung injury between normoxic (n=4) and hypoxic (n=3) animals

Figure 4.2.4.1

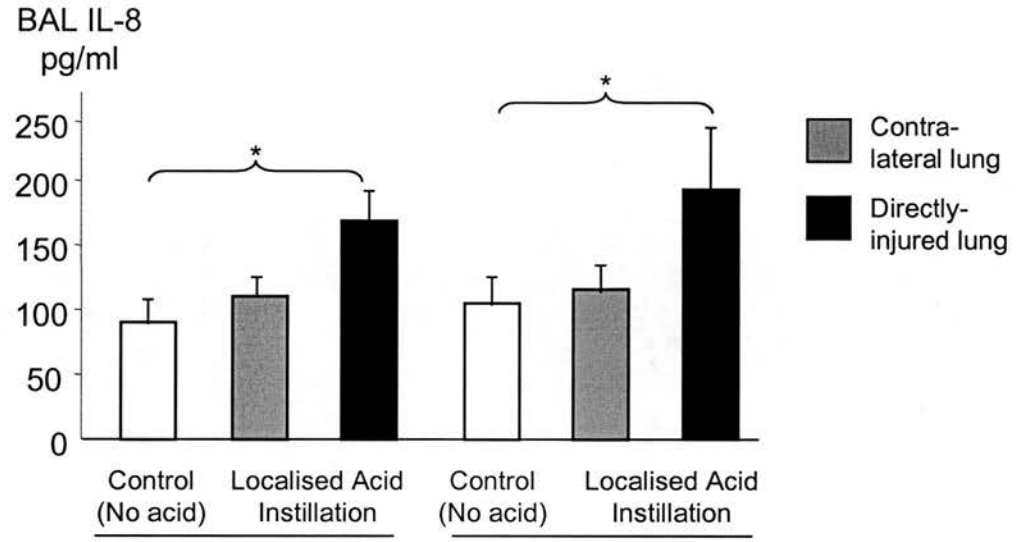
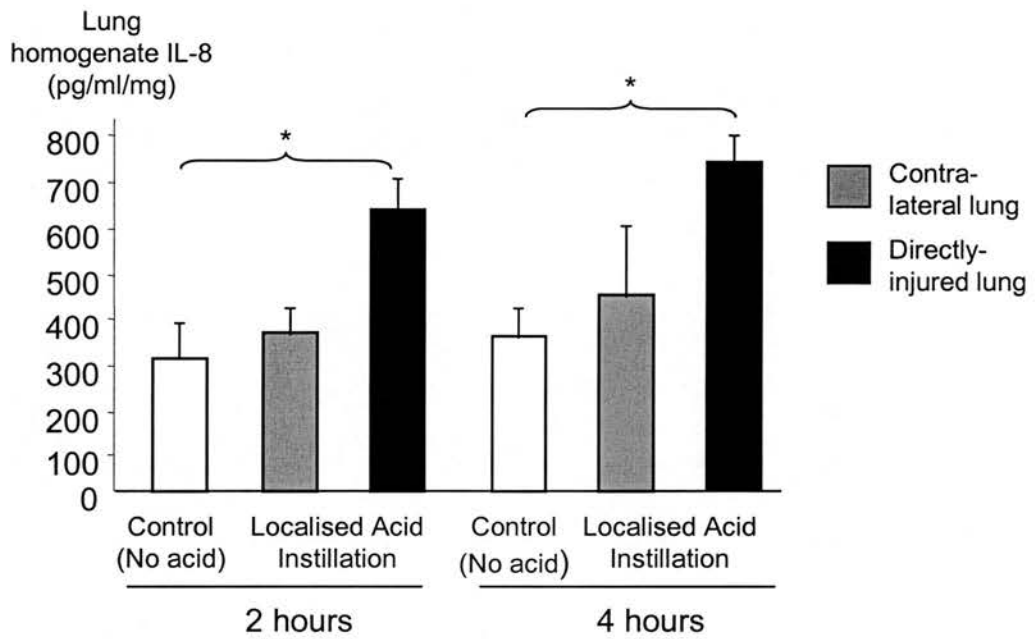


Figure 4.2.4.2



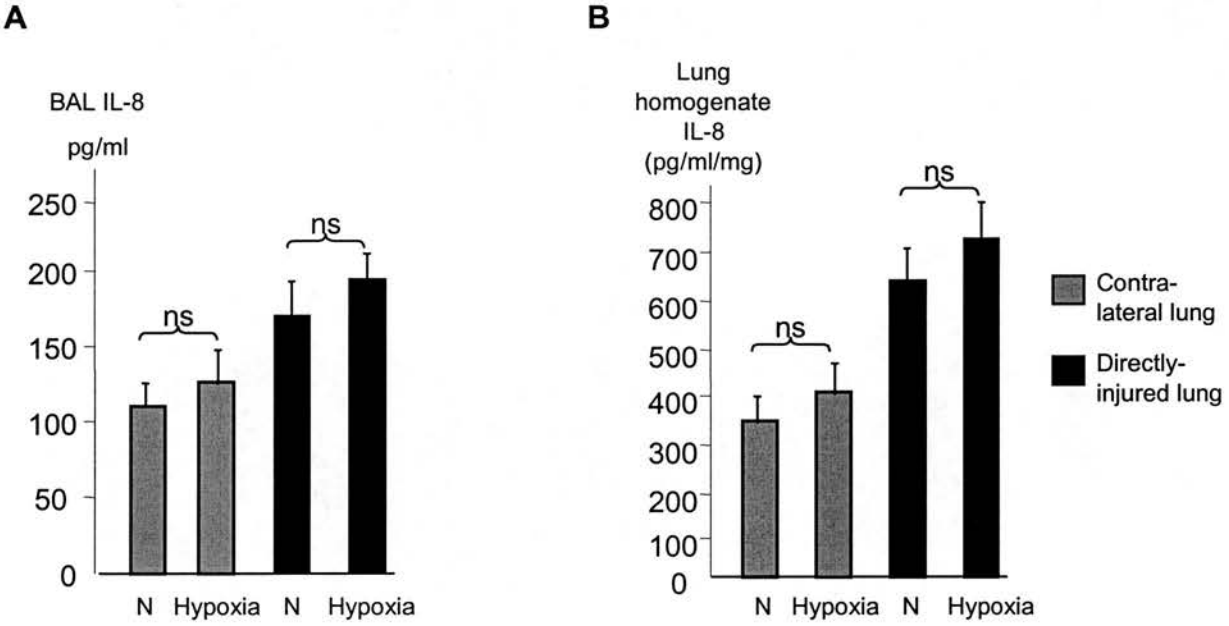
Legend for Figures 4.2.4.1 and 4.2.4.2. Direct and indirect effect of localised acid instillation on intrapulmonary IL-8 generation.

Localised instillation of acid followed by ventilation under normoxic conditions significantly increased BAL IL-8 (**Figure 4.2.4.1**) and lung homogenate IL-8 (**Figure 4.2.4.1**) in the directly injured lung at 2 hours (n=4), and 4 hours (n=6) compared to control ventilated animals (n=3) without acid instillation. There was no significant effect on intrapulmonary IL-8 in the contralateral lungs of acid-instilled animals *P<0.02.

4.2.5. The effect of acute hypoxia on IL-8 protein generation in the acid-induced lung injury model

Hypoxia had no significant effect on IL-8 protein expression in directly injured or contralateral lungs (**Figures 4.2.5**)

Figure 4.2.5



Legend for Figure 4.2.5. IL-8 protein in BAL and lung homogenate in normoxic and hypoxic animals. There was no significant (ns) difference in IL-8 protein expression between hypoxia and normoxic (N) animals in BAL (**A**) or lung homogenate (**B**). Results are expressed as mean ± SEM. n=4 normoxic and n=3 hypoxic animals.

4.2.6. The effect of acute hypoxia on IL-8 mRNA expression in the acid-induced lung injury model

Interleukin-8 mRNA expression in lung homogenate was determined by RT-PCR. In the acid-injured lungs, IL-8 mRNA expression was 2.1 fold greater in hypoxic compared to normoxic animals. Hypoxia had no effect on IL-8 mRNA expression in the contralateral lung (**Figures 4.2.6**).

Figure 4.2.6

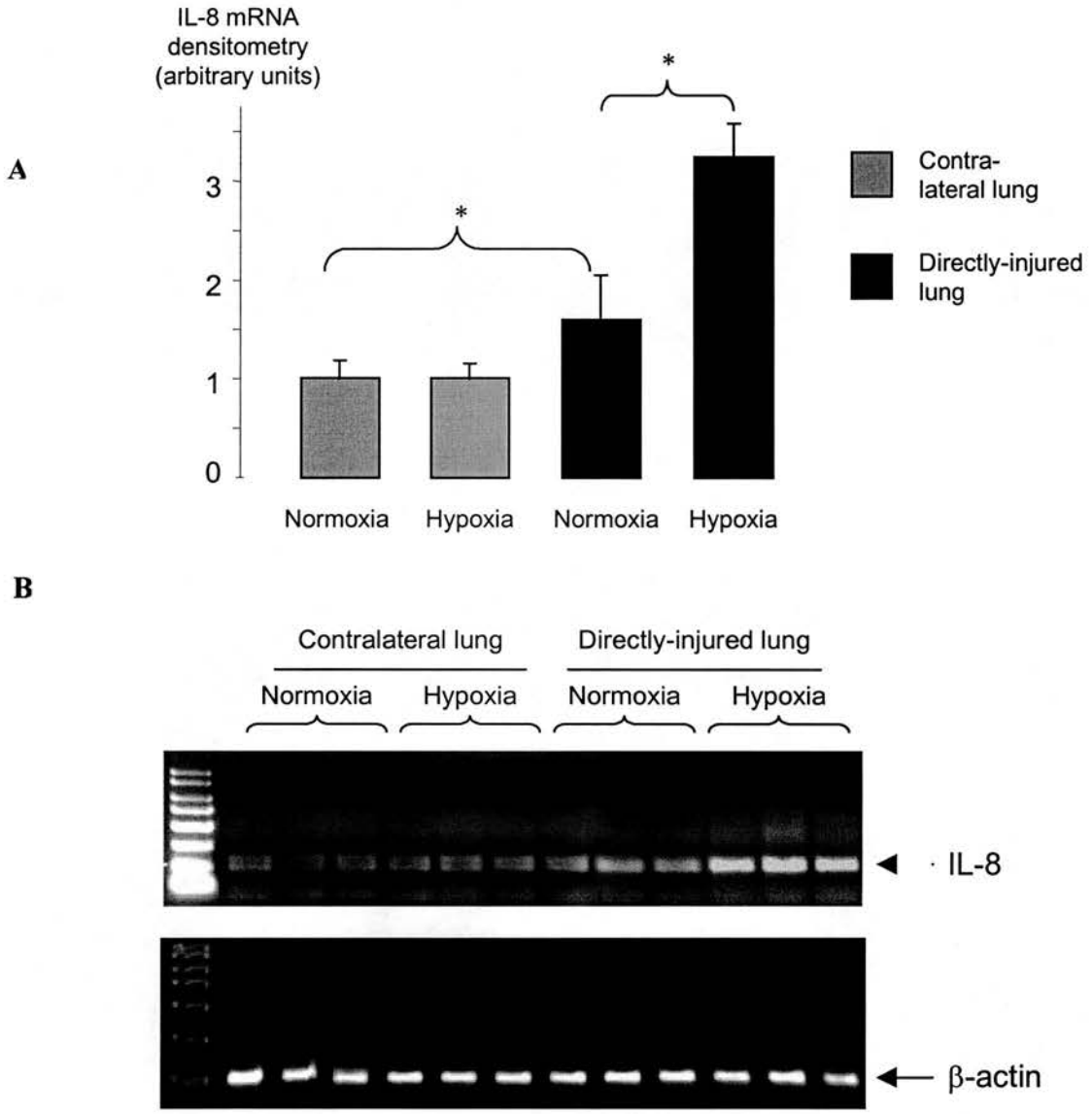


Figure 4.2.6. IL-8 mRNA expression in acid-instilled and contralateral lungs in normoxic and hypoxic animals. Acid-instillation significantly increased IL-8 mRNA expression in lung homogenate from directly-injured but not contralateral lungs. Hypoxia further upregulated IL-8 mRNA expression in the directly-injured lungs. IL-8 densitometry (**A**) was corrected for β-actin expression from the PCR gel shown (**B**). Results are expressed as mean ± SEM. *P<0.05

4.2.7. The effect hypoxia / hyperoxia and hypoxia / controlled reoxygenation on acute lung injury and IL-8 generation

Having found that hypoxia significantly increased intrapulmonary IL-8 mRNA expression but not protein expression or measures of lung injury, a further potential 'hit' was introduced with reoxygenation to hyperoxic levels (FiO_2 100 %, arterial PO_2 ~ 55 KPa mmHg), or to relatively normoxic levels (controlled reoxygenation (CR), FiO_2 ~40%, arterial PO_2 ~15 KPa). Following acid-instillation, animals were ventilated for 4 hours under normoxic or 2 hr hypoxic / 2 hr hyperoxic, or 2 hours hypoxia / 2 hours CR conditions (**Figure 4.2.7**)

Lung protein leak and BAL neutrophil data is summarised in **Table 4.2.7**. In the directly injured lung, compared to normoxia, hypoxia / hyperoxia resulted in a 2-fold increase in lung protein leak. There was also a trend for greater number of BAL neutrophils though this did not reach statistical significance (P=0.07).

In the contra-lateral lung, hypoxia / hyperoxia resulted 4.8-fold increase in lung protein leak compared to normoxic controls. BAL levels of neutrophils were not significantly different to controls. Hypoxia / CR significantly attenuated lung protein leak in both directly-injured and contralateral lungs and reduced BAL neutrophils in the directly-injured lung (P=0.06)

In the directly injured lung, hypoxia / hyperoxia resulted in significantly increased IL-8 protein in BAL fluid and lung homogenate compared to normoxia. In the contralateral lung, IL-8 levels were not significantly higher than controls in BAL fluid or lung homogenate. As with lung leak, controlled reoxygenation significantly attenuated IL-8 generation (**Figure 4.2.7**)

Serum IL-8 levels were undetectable at all time points.

Table 4.2.7

	Contralateral lung			Directly-injured lung		
	Normoxia	Hypoxia / Hyperoxia	Hypoxia / CR	Normoxia	Hypoxia / Hyperoxia	Hypoxia / CR
Lung protein leak (mls)	0.038 ± 0.01	0.184 ±0.09*	0.07 ± 0.03	0.44 ± 0.8	0.82 ± 0.15†	0.46 ± 0.11
BAL PMN (%)	1.2 ± 0.8	2.1± 0.4	1.8 ± 1.1	35 ± 10	48 ± 17	31 ± 14
BAL PMN (cells x10^5 / 10 ml BAL fluid)	0.18 ± 0.4	0.29 ± 0.16	0.36 ± 0.3	22.5 ± 4	31 ± 5‡	20.2 ± 3

Table 4.2.7. Effect of hypoxia / hyperoxia and hypoxia / CR (controlled reoxygenation) on lung injury

Data are presented as means ± SEM, except BAL PMN (%) which is represented by median ± SD. N=6 normoxia, n=6 hypoxia / hyperoxia, n=4 hypoxia / CR

*P<0.05 compared to contralateral lung in 4 hour normoxic and hypoxic/ CR groups.

†P<0.05 compared to directly-injured lung in 4 hour normoxic and hypoxic / CR group

‡P=0.07 compared to directly injured lung in 4 hour normoxic group and P=0.06 compared to hypoxic / CR group

Figure 4.2.7

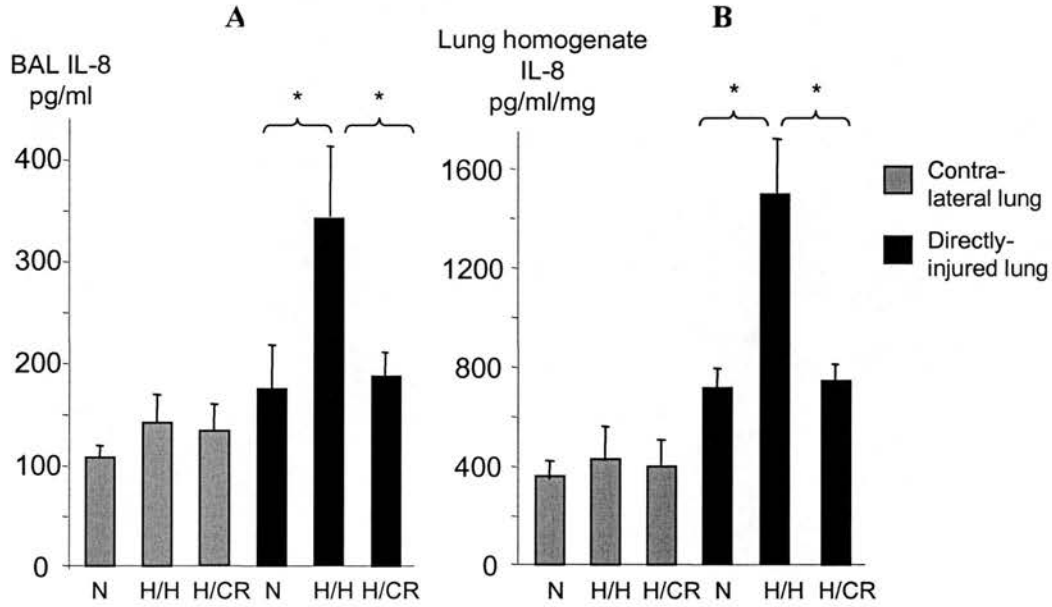


Figure 4.2.7. The effect of hypoxia / hyperoxia and hypoxia / controlled reoxygenation on IL-8 generation in directly injured and contralateral lungs. In the directly injured lung, hypoxia / hyperoxia (H/H) significantly increased IL-8 levels in both BAL fluid (**A**) and lung homogenate (**B**), compared to normoxia (N). Hypoxia / controlled reoxygenation (H/CR) significantly attenuated this effect. Varying oxygenation had no effect on IL-8 generation in the contralateral lung. Results are expressed as mean ± SEM. n=6 normoxia, n=6 H/H, n=4 H/CR. *P<0.02

4.3. DISCUSSION

In these studies, a novel model of acute lung injury was developed in which to study the effects of acute hypoxia and hyperoxia on intrapulmonary IL-8 generation. Localised acid instillation induced acute lung injury with raised IL-8, lung protein leak and neutrophil influx in the directly injured lobe, but no injury in the contralateral lung. Acute hypoxaemia, achieved by reducing FiO_2, was associated with increased IL-8 mRNA but not protein expression in the acid-injured lung. Acute hyperoxia following hypoxia generated raised intrapulmonary IL-8 protein expression and exacerbated lung leak, and both were attenuated if hyperoxia was substituted with controlled reoxygenation. In the contralateral lung, a model of potential indirect lung injury, changes in oxygenation did not induce IL-8 upregulation, but hypoxia / hyperoxia resulted in significant lung leak.

Direct instillation of acid into the left lower lobe induced acute lung injury as characterised by increased neutrophils in airspaces and alveolar-capillary protein leak (lung leak). Localised lung injury was apparent by 2 hours following acid instillation and was associated with raised intrapulmonary and BAL IL-8. As with ARDS, the neutrophil is thought to play a significant role in acid-induced lung injury (Folkesson et al., 1995; Goldman et al., 1992; Knight et al., 1992). Our studies were limited to the early phase post-injury. Hence, they do not serve to investigate the role of increased intrapulmonary IL-8 generation on the progression of acute lung injury to ARDS. However, there is evidence that IL-8 plays a central role in the pathogenesis of acute lung injury in rabbits. Targeting IL-8 with an anti-IL-8 antibody has been shown to attenuate rabbit lung injury secondary to acid-aspiration (Folkesson et al., 1995; Modelska et al., 1999), ischaemia / reperfusion (Sekido et al., 1993) and pulmonary re-expansion (Nakamura et al., 2000). Based on these studies, it is likely that raised intrapulmonary IL-8 in our multiple-hit model of lung injury would have a prominent role in progression of lung injury.

It is accepted that reducing FiO_2 is not a relevant mechanism for causing hypoxia in clinical situations associated with acute lung injury, except perhaps in the lung injury associated with high altitude (high altitude pulmonary oedema, HAPE). The closest clinical analogy to the model presented in this study is aspiration of gastric contents, an

event usually associated with impaired consciousness (Adnet and Baud, 1996; Marik, 2001). Hypoventilation would be a more clinically relevant cause of alveolar hypoxia in this context. However, it is also known that ventilation strategies may influence lung injury. Ventilation-associated stretch or barotrauma is known to influence intrapulmonary cytokine generation (Tremblay et al., 1997) and induce lung injury (Kolobow et al., 1987; Tsuno et al., 1991; Dos Santos and Slutsky, 2000). Since the principle hypothesis being tested in the presented studies concerned the influence of oxygenation on intrapulmonary IL-8 generation, it was thought important to minimise the effects of these variables in the model. Hence, care was taken to manipulate oxygenation only, whilst maintaining baseline ventilatory and physiological parameters.

Through reducing FiO_2 to 17%, a stable arterial PO_2 of 5 KPa was achieved for 2 hours without altering ventilation parameters and whilst maintaining normal arterial pH and PCO_2. In healthy lungs, this strategy would result in a mean alveolar PO_2 of ~10 KPa (**Chapter 1.4**). Thus, it might reasonably be argued that alveolar cells were not rendered significantly hypoxic in this model. In the non-injured right lung, this may be one explanation for the lack of effect of 'hypoxia' on intrapulmonary IL-8 generation. However, in the acid-injured left lower lobe, in which there was interstitial oedema and airspace flooding, a significant degree of alveolar hypoxia may have been achieved. In the absence of a cellular marker for hypoxia, this is not proven. The finding that IL-8 mRNA expression in acid-injured lung was significantly higher in hypoxic compared to normoxic animals, ventilated under identical conditions with only FiO_2 varied, suggests a role for hypoxia in intrapulmonary IL-8 generation.

In the context of a multi-hit model of inflammation, one interpretation of this data is that hypoxia, through generation of increased IL-8 mRNA, acts as a proinflammatory hit *in vivo*. A further hit, namely hyperoxia, was required to significantly increased IL-8 protein expression in the acid-injured lung, in which there was also a trend (P=0.07) towards increased BAL neutrophil count. In the contralateral lung, hypoxia / hyperoxia did not influence IL-8 expression. Since our experiments did not include a '4 hour hyperoxia'

group, it cannot be determined if hypoxia / hyperoxia is a more potent stimulus for IL-8 generation than hyperoxia alone, as was demonstrated *in-vitro*. However, our primary aim was to mimic a typical clinical scenario in which acute hypoxia is followed by acute hyperoxia, and to determine if this stimulus is sufficient to raise intrapulmonary IL-8 levels. The data suggests that in our model, hypoxia / hyperoxia is able to rapidly upregulate intrapulmonary IL-8 in the setting of direct lung injury.

Many studies have demonstrated that hyperoxia induces lung leak, but only following prolonged (>24 hours) exposure (Shasby et al., 1982; Crapo 1986; Adawi A et al., 1998; Corne et al., 2000). Such studies have been performed in non-ventilated animals and in the absence of co-existing localised or distant injury. In contrast, our data shows that compared to normoxic controls, acute hypoxia / hyperoxia in ventilated animals with localised acute lung injury significantly increased lung leak in both the acid-injured and contralateral lung. Thus, acute hyperoxia may be injurious, but only as part of one or more inflammatory 'hits' (Smith et al., 1990). In a rat model, the combination of 90% O_2 and intravenous bleomycin induced acute lung injury by 4 hours, though neither agent was injurious in isolation at this time point (Hay et al., 1987)

Our finding of increased lung leak in the contralateral lung is of interest. The absence of associated raised IL-8 or BAL neutrophils suggests lung leak occurred via a mechanism that may be neutrophil-independent. It has been suggested that increased reactive oxygen species (ROS) direct injure alveolar epithelial cells and play a role in hyperoxia-induced lung injury (Crapo, 1986; Folz et al., 1999; Nader-Djalal et al., 1998) The lung leak as measured in our model represents passage of albumin into the alveolar airspace. This provides a potential mechanism for exacerbating lung injury, since circulating inflammatory mediators, such as cytokines and endotoxin, may also pass into the alveolar space and further activate inflammatory cells including the alveolar macrophage. It is acknowledged however that a degree of albumin leak may have been induced by the BAL procedure itself, as has been described in human studies (Ward et al., 1992; Baughman RP, 1997). Hence the absolute degree of albumin leak into the alveolar airspaces must be interpreted with some caution.

Our finding that increased IL-8 and lung leak was attenuated to baseline levels if acute hypoxia was followed by controlled (FiO_2 ~40%) rather than hyperoxic (FiO_2 100%) reoxygenation may be of clinical relevance. In the acute resuscitation scenario, the delivery of 80-100% oxygen to hypoxic critically-ill patients is standard practice. Our animal data supports the hypothesis that acute hyperoxia in this setting may exacerbate sub-clinical acute lung injury and induce intra-pulmonary IL-8 generation. Resuscitating hypoxic patients with controlled oxygen to physiological levels may attenuate the intrapulmonary inflammatory response in the early at-risk period of ARDS. However, this strategy would need to be countered with the potentially lethal consequences of acute severe tissue hypoxia.

With regard to our clinical observations in at-risk patients, it is acknowledged that these animal studies do not fully explain our finding of early raised intrapulmonary IL-8 generation in those that progress to ARDS. The controlled strategy for inducing hypoxia and hyperoxia only through varying FiO_2 served to exclude factors such as atelectasis and barotrauma. Both phenomena are however likely to be relevant in the acute clinical setting and may contribute to intrapulmonary inflammation.

CHAPTER 5

THE REGULATION OF IL-8 GENE EXPRESSION BY ACUTE HYPOXIA IN MACROPHAGES

5.1 INTRODUCTION

In the previous chapters, it has been shown that 2 hours hypoxia significantly increased IL-8 protein secretion from monocyte-derived macrophages. By 4 hours, IL-8 secretion was no different to normoxic controls, but steady-state IL-8 mRNA expression was increased. In rabbit alveolar macrophages, and in rabbit acid-injured lungs, acute hypoxia was associated with increased IL-8 mRNA expression, but not increased protein.

The 5'-flanking DNA of the IL-8 gene contains potential binding sites for a number of transcription factors (**Figure 5.1**). However, based upon DNAse footprinting and transcriptional fusion studies, the 130 bp sequence immediately upstream of the TATA box has been shown to be sufficient for maximal transcriptional responses to most pro-inflammatory mediators (Mukaida et al., 1994; Yasumoto et al., 1992; Nakamura et al., 1991). Within this region, lie functional binding sites for activator protein-1, (AP-1), CCAAT/Enhancer binding protein-β (C/EBP-β, also termed nuclear factor-IL-6, NF-IL-6) and NF-κB. A putative HIF-1 binding site located 3' of the TATA box and immediately upstream of the transcription initiation site is discussed in **Chapter 6**.

In this chapter, I aimed to further determine the molecular mechanisms involved in acute hypoxia – induced IL-8 mRNA expression in monocyte-derived macrophages. In particular, the regulation of transcription factors AP-1, C/EBP-β and NF-kB by hypoxia were studied and compared to the effects of a classical proinflammatory stimulus, LPS.

Figure 5.1

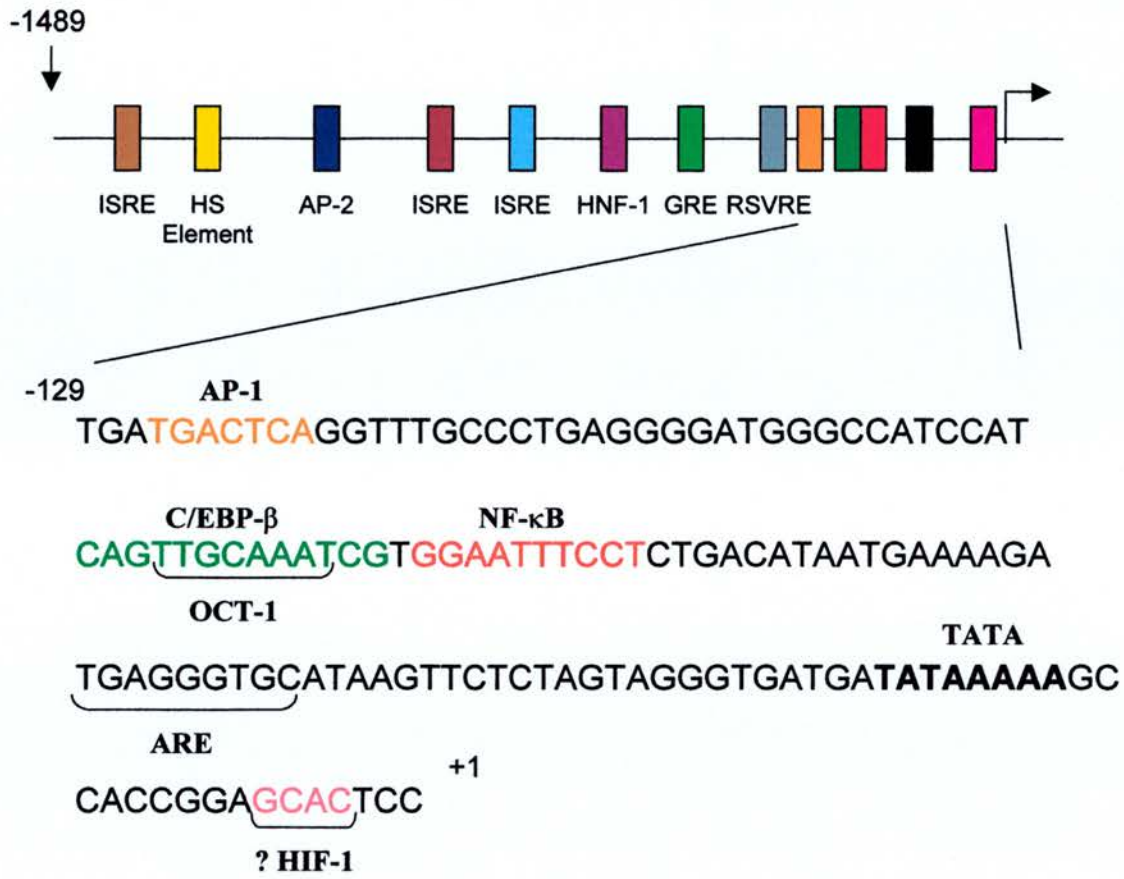


Figure 5.1. The IL-8 gene promoter region with transcription factor binding sites.
Several transcription factor binding sites have been identified within 1.5 Kb 5' region of the IL-8 gene. However, the 130 bp region upstream of the TATA box is sufficient to induce maximal gene transcription for most stimuli. This region contains the binding sites for AP-1, C/EBP-β and NF-κB.
Additional sites of likely functional significance are:
i) An Oct-1 element that overlaps with the C/EBP-β site and has been shown to repress IL-8 transcription (Wu et al., 1997)
ii) An AP-2 element (GAGTGGCC site located -868 to -876, consensus GCCNNNGGC or GSSWGSC) which is required for the IL-8 response to the *Pseudomonas* autoinducer N-3-oxododecanoyl homoserine lactone (Smith et al., 2001)
iii) An interferon stimulated response element (IRSE) – (consensus GAAASYGAAASY or GAAAASYGAAAASY). A number of GAAA sites are harboured upstream, including one within the RSVRE
iv) A respiratory syncytial virus-induced element (RSVRE) located -132 to -162 (ACTCCGTATTTGATAAGGAACAAATAGGAAG) (Casola et al., 2001). This sequence contains an IRSE and a putative HNF-3 (=IRS, insulin response sequence).

There are several putative binding sites, the functional significance of which is unknown. These include ones for hepatic nuclear factor-1 (HNF-1 consensus GGTTAATNATTAAC with ATTAAC identified -376 to –381), glucocorticoid response element (GRE consensus GGTACANNNTGTTCT, with TGTTCT identified -330 to –325), heat shock element (HSE consensus generally NGAAN often as multiple copies as seen in the 5' IL-8 promoter) and early growth factor-1 (Egr-1 consensus sequence GCG(G/T)GGCG, with CCGTAAGGGGAGG located on the antisense strand at -1350 to 1361). Finally, a putative HIF-1 binding site is located on the antisense strand immediately 5' of the transcription initiation site and downstream of the TATA box.

5.2 RESULTS

5.2.1 Hypoxia increases IL-8 mRNA in macrophages.

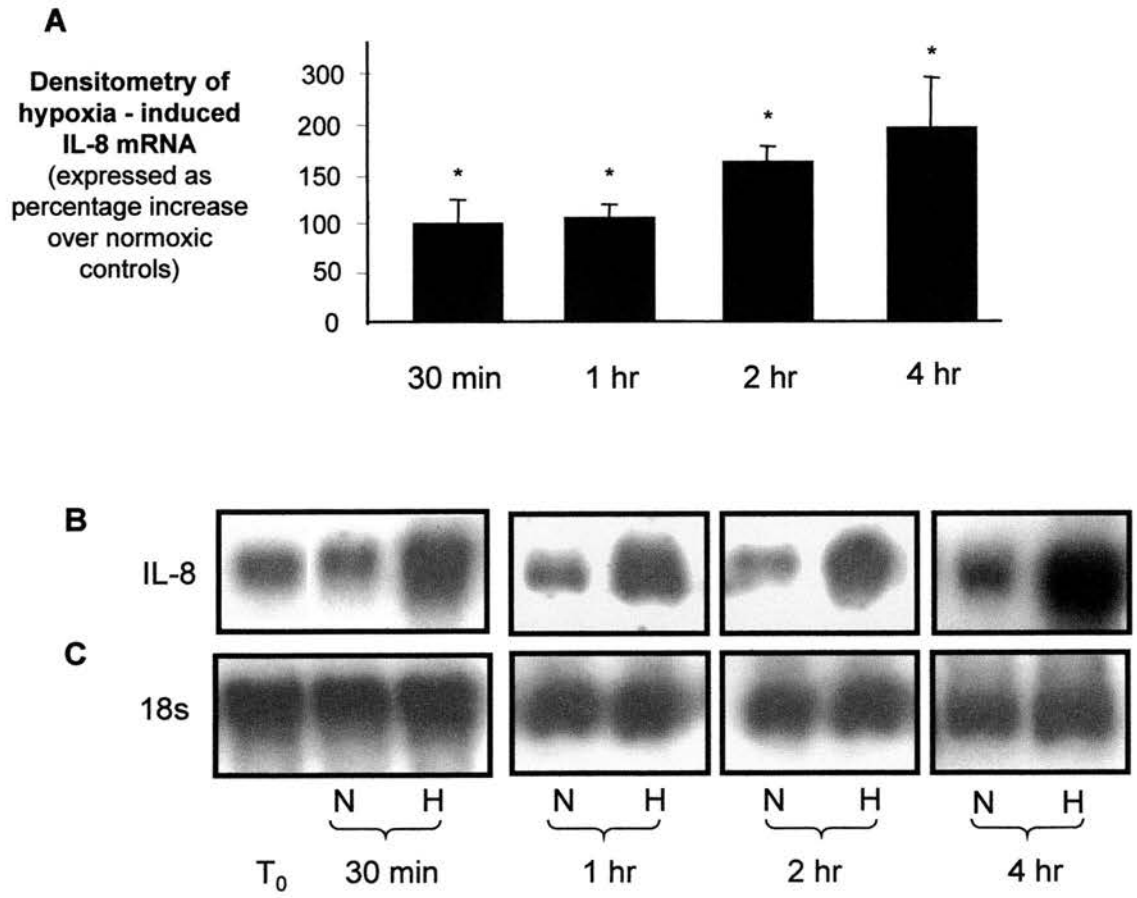
Macrophage IL-8 mRNA expression was measured by northern blotting. Hypoxia significantly increased steady-state IL-8 mRNA levels by 30 min, 1 hour, 2 hours and 4 hours (90±25, 104±19, 157±19 and 318±91 respectively, expressed as percentage increase over normoxic controls. $P<0.01$) (**Figure. 5.2.1**). An increase in mRNA transcription and / or mRNA stability may account for the raised steady-state expression of IL-8 under hypoxic conditions. However, the finding that within 30 min of hypoxic exposure, IL-8 mRNA levels were 88±19 % higher than at T_0 (macrophages cultured over 5 days and RNA extracted), suggests increased gene transcription at least plays a part in raising IL-8 mRNA expression.

Three transcription factors, NF-κB, AP-1 and C/EBP-β have been implicated in IL-8 regulation by a range of stimuli and in a variety of cell lines (Roebuck, 1999). Using EMSA, nuclear levels of these proteins were determined following acute hypoxic exposure. For comparison, macrophages were also stimulated with LPS, an archetypal proinflammatory stimulus shown in **Chapter 3** to be a potent inducer of IL-8 protein expression from macrophages.

5.2.2. Hypoxia does not increase nuclear NF-κB levels

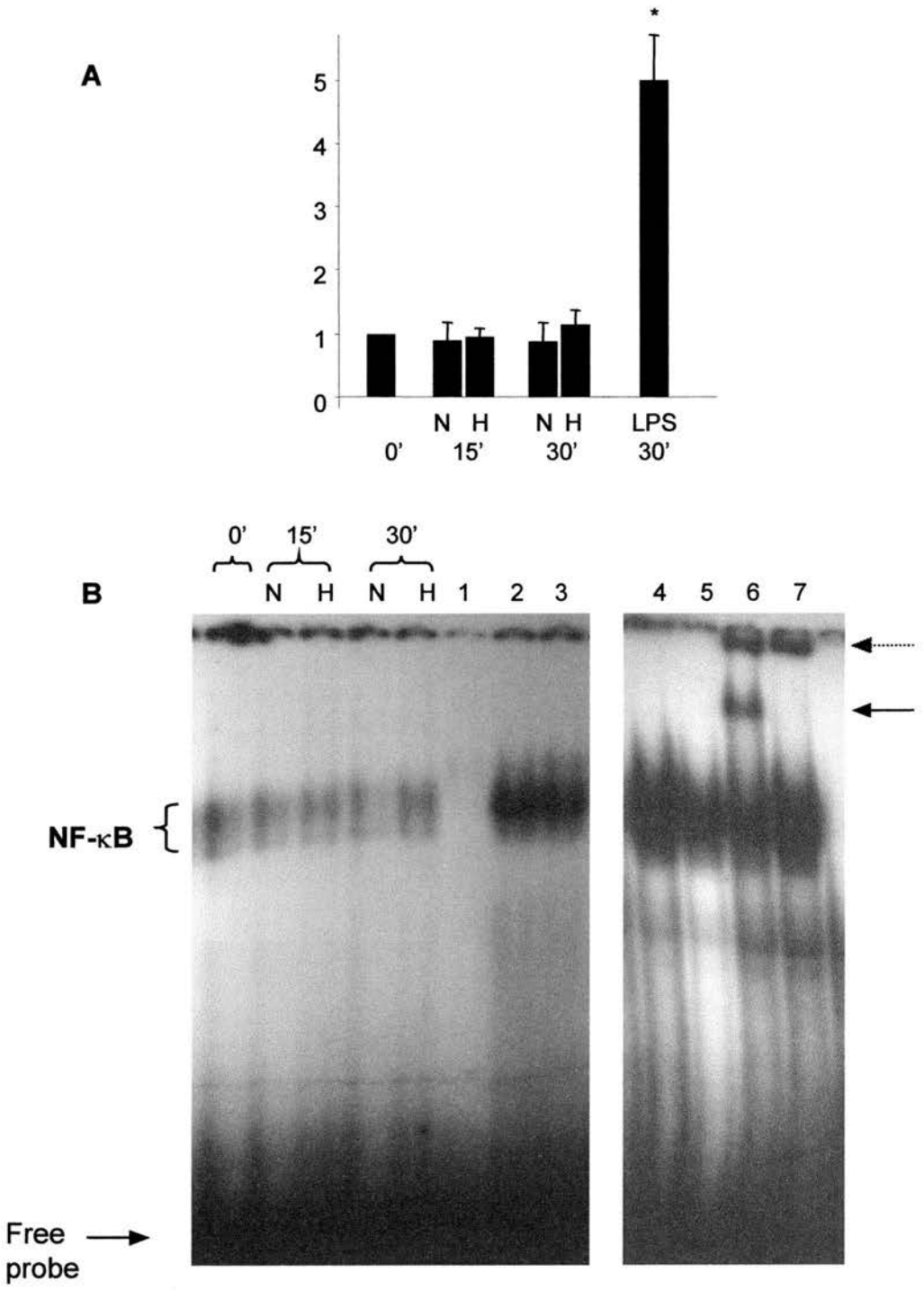
Hypoxia for up to 30 min did not increase nuclear NF-κB above normoxic levels. LPS by contrast increased NF-κB levels by 322±34% by 30 min (**Figure 5.2.2**). The specificity of the observed band was confirmed by non-radiolabelled (cold) NF-κB competition protein binding. A supershift assay with LPS-treated macrophages revealed two supershifted bands with a p50 antibody and one with a p65 antibody, suggesting the activated NF-κB complex contained both subunits.

Figure 5.2.1



Legend for Figure. 5.2.1. Effect of hypoxia on Macrophage IL-8 mRNA expression. Steady-state mRNA expression was measured by northern blotting. Hypoxia significantly increased steady-state IL-8 mRNA levels by 30 min, 1 hour, 2 hours and 4 hours (90±25, 104±19, 157±19 and 318±91 respectively, expressed as percentage increase over normoxic controls. ($P<0.01$). Each value in 5.2.1**A** represents mean ± SEM of >3 experiments on n=4 donors. The northern blots shown are from macrophages derived from one donor on two separate occasions. After probing for IL-8 (5.2.1**B**), blots were stripped and re-probed for 18s housekeeping gene to assess RNA loading (5.2.1**C**).

Figure 5.2.2



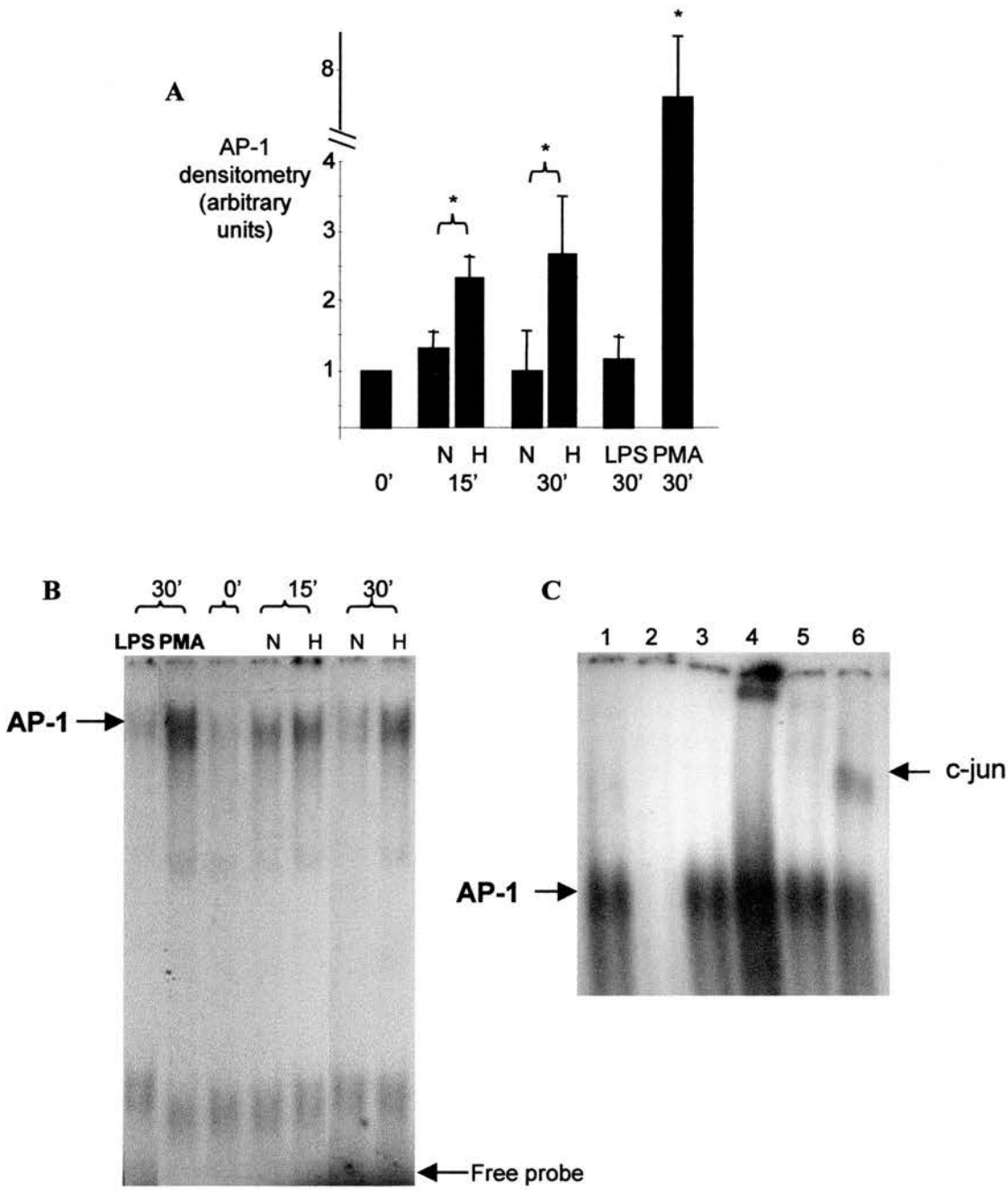
Legend for Figure 5.2.2. Hypoxia does not increase nuclear NF-κB levels.
Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30min and nuclear levels of NF-κB determined by EMSA. Hypoxia did not increase nuclear NF-κB abundance above basal levels. LPS (1 μg/ml for 30 min) strongly induced nuclear NF-κB activation. Each value in 5.2.2**A** represents mean ± SEM from n=4 separate experiments (*P<0.01).

Legend for Figure 5.2.2. continued
5.2.2 **B.** A representative EMSA is shown including a competition assay. Lane 3 contains 4 μg of protein from LPS-stimulated macrophages. Lane 2 as 3 with the addition of 100 X excess cold non-competitor (oligonucleotide with mutated NF-κB binding site). Lane 1, 100 X excess cold NF-κB competitor.
5.2.2 **C.** A supershift assay further defines this LPS-induced band. Lane 4 contains nuclear extracts from LPS-stimulated macrophages (as Lane 1). Subsequent lanes with addition of 2 μl control rabbit serum (Lane 5), p50 antibody (Lane 6 , revealing two distinct supershifted bands [broken arrow]) or p65 antibody (Lane 7, revealing one supershifted band [solid arrow]). This implies that the presence both p50 and p65 subunits within the NF–κB complex, but the precise dimerisation characteristics are not identifiable from this study. The supershift is representative of n=2 studies.

5.2.3. Hypoxia increases nuclear AP-1 levels

Hypoxia increased nuclear AP-1 levels by 67±11% and 130±34% at 15 and 30 min respectively as measured by densitometry ($P < 0.02$, n=4) (**Figure 5.2.3**). LPS (1 μg/ml) did not significantly affect AP-1 levels by 30 min of exposure. Phorbol myristate acetate (PMA) (100 nM) was used as an alternative positive control, and was found to increase AP-1 levels by 610±95 % by 30 min compared to normoxia. The specificity of the AP-1 band was confirmed by competition assay. Co-incubation with c-Jun, but not c-Fos antibody, demonstrated a supershifted band.

Figure 5.2.3



Legend for Figure 5.2.3 Hypoxia increases nuclear AP-1 levels

Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30 min and nuclear levels of AP-1 determined by EMSA.

Compared to normoxic controls, hypoxia significantly increased AP-1 levels by 15 and 30 min as measured by densitometry. Each value in 5.2.3**A** represents mean ± SEM of n=4 separate experiments. (*P=0.02). LPS (1 μg/ml) for 30 min does not induce AP-1 activation. PMA (100 nM), a known upregulator of AP-1 was used as a positive control.

Legend for Figure 5.2.3 continued.
A representative EMSA is shown (5.2.3**B**). The specificity and nature of the observed AP-1 band is addressed by competition and supershift assay. Lane 1 contains 4 μg nuclear protein from hypoxic macrophages incubated with end-labelled AP-1 oligonucleotide probe only. Subsequent lanes are as Lane 1 with the addition of 100 X excess cold AP-1 competitor (Lane 2), 100 X excess cold non-competitor (oligonucleotide with mutated AP-1 binding site (Lane 3), 2 μl rabbit serum (Lane 4 with non-specific band), c-Fos antibody (Lane 5) or c-Jun antibody (Lane 6 with supershifted band).

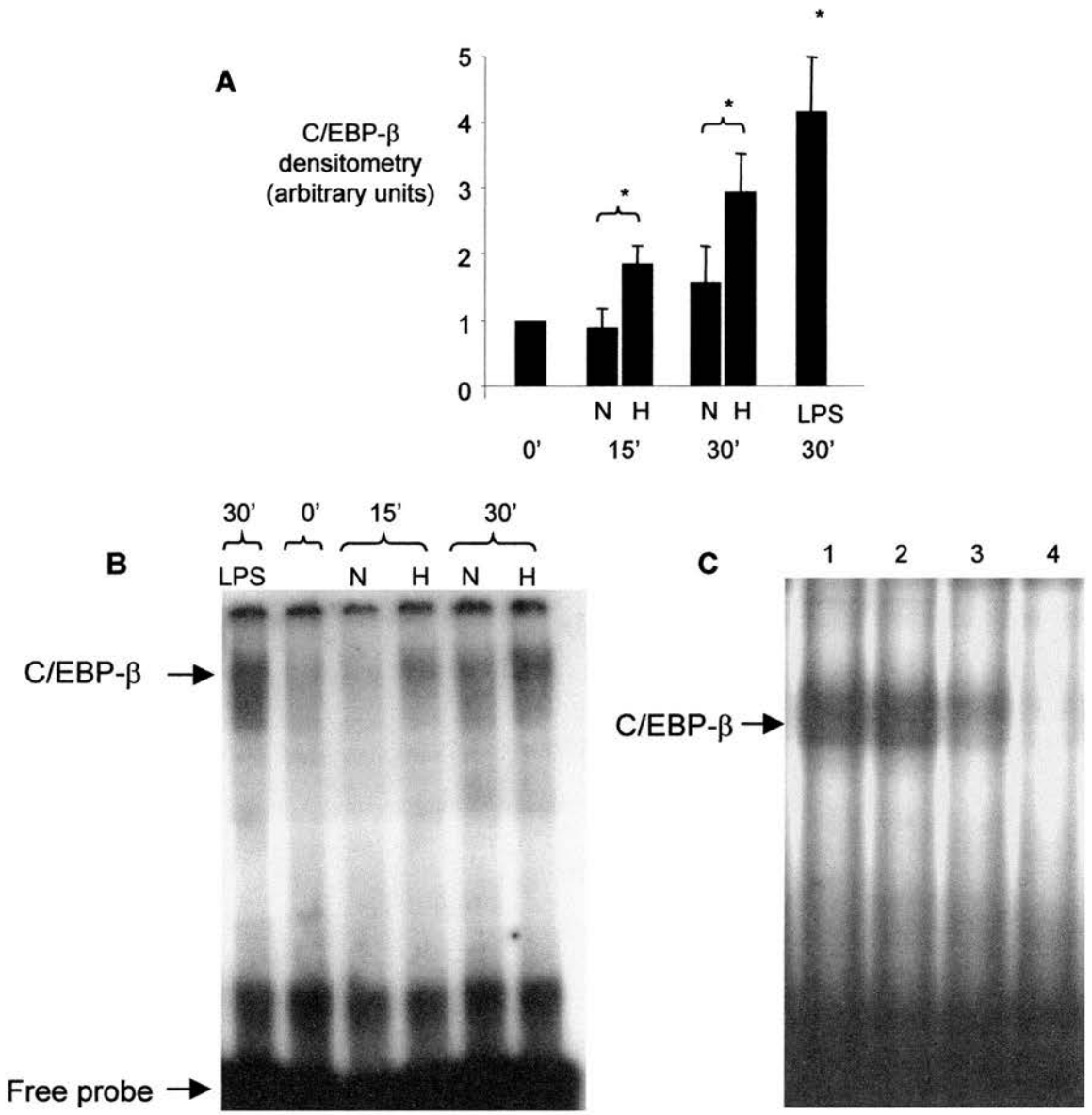
5.2.4. Hypoxia increases nuclear C/EBP-β levels

Hypoxia increased nuclear C/EBP-β levels by 79±9% and 82±18% at 15 and 30 min respectively as measured by densitometry (**Figure 5.2.4**). By comparison, LPS induced a 160±26 % increase in levels by 30 min compared to normoxic controls ($P < 0.02$, n=4). The specificity of the band-shift observed was confirmed by competition assay, which showed no affect with excess cold non-competitor and abolishment with excess cold competitor.

5.2.5. N-acetylcysteine inhibits IL-8 mRNA expression in association with reduced nuclear AP-1, but not basal NF-κB, activation.

The finding that hypoxia increased nuclear AP-1 and C/EBP-β, but not NF-κB, nuclear expression was unexpected. Most, though not all, inducers of IL-8 gene transcription are associated with NF-κB activation (Roebuck et al., 1999). It is apparent however that the macrophages in the present studies expressed basal levels of NF-κB under normoxic conditions without stimulation (**Figures 5.2.2 and 5.2.5**). N-acetylcysteine (NAC), a thiol anti-oxidant, has been shown to inhibit inflammatory gene transcription (Pahan et al., 1998; Sprong et al., 1998) and may have differential effects on AP-1 and NF-κB activation (Schreck et al., 1991). Macrophages were co-incubated under hypoxic conditions with NAC. Hypoxia-induced IL-8 mRNA expression at 30 min was attenuated by NAC 0.5 mM and 5 mM. Basal nuclear NF-κB expression was unchanged, but nuclear AP-1 abundance was reduced at these doses (**Figure 5.2.5**). The pH of the medium following after addition of NAC was adjusted to 7.0 with sodium hydoxide prior to cell culture.

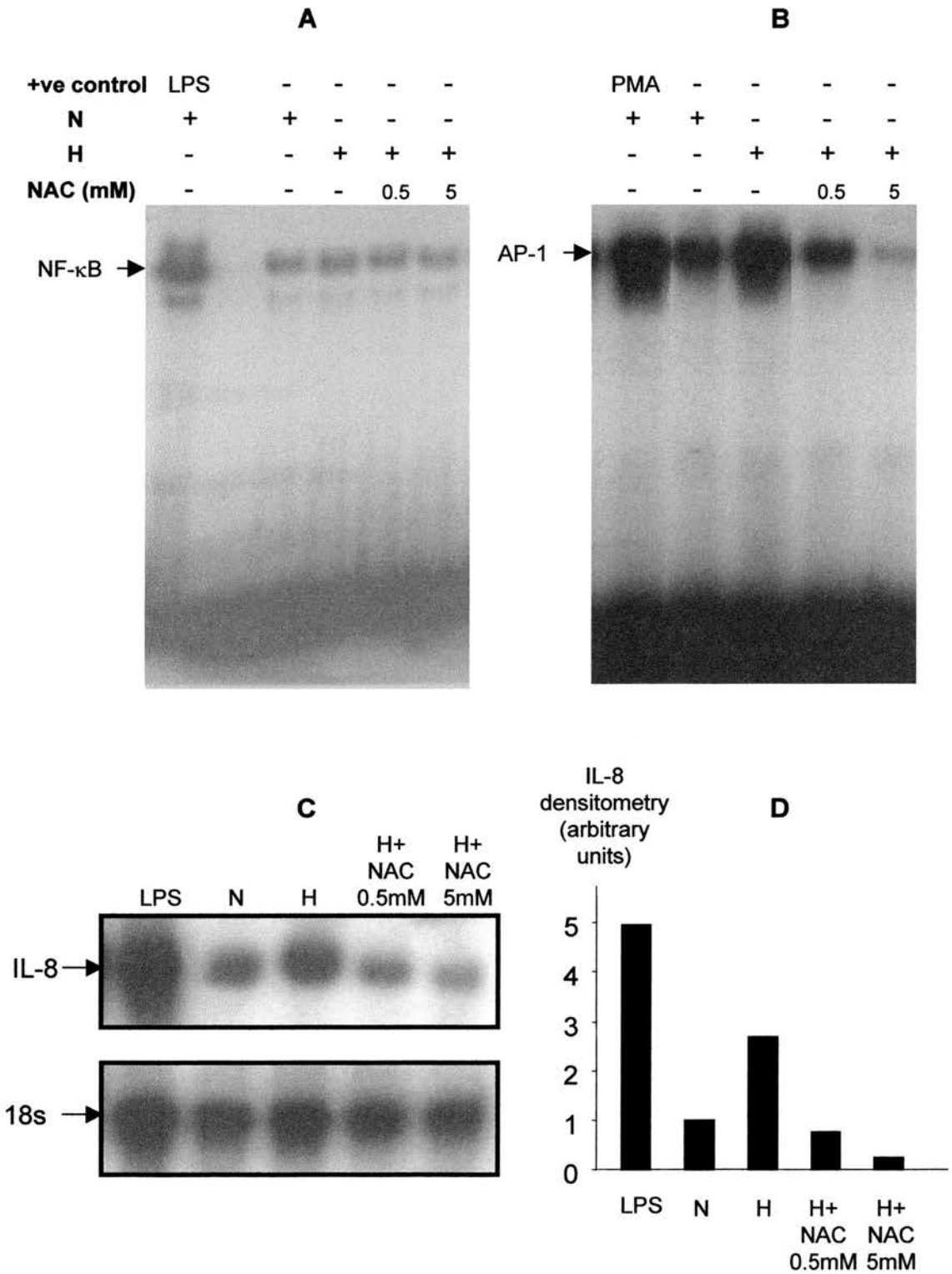
Figure 5.2.4



Legend for Figure 5.2.4. Hypoxia increases nuclear C/EBP-β levels. Macrophages were incubated under normoxic (N) or hypoxic (H) conditions for up to 30min and nuclear levels of C/EBP-β determined by EMSA. Hypoxia significantly increased C/EBP-β nuclear abundance by 15 and 30 min as measured by densitometry. Each value in 5.2.4 **A** represents mean ± SEM of n=4 separate experiments (*P<0.02). LPS (1 μg/ml for 30 min) induces a 160±26% increase compared to normoxic controls.

A representative EMSA is shown in 5.2.4**B**. The specificity of the observed C/EBP-β band was addressed by competition assay (5.2.4**C**). Lane 1 contains 4 μg of protein from hypoxic macrophages incubated with end-labelled C/EBP-β oligonucleotide probe only. Lane 2 as Lane 1 with the addition of 100 X excess cold C/EBP non-competitor. Lanes 3, 100 X excess cold C/EBP-β competitor (oligonucleotide with mutated C/EBP-β binding site). Lane 4, 1000 X excess cold C/EBP-β competitor.

Figure 5.2.5



Legend for Figure 5.2.5. N-Acetylcysteine inhibits hypoxic upregulation of IL-8 mRNA in association with reduced nuclear AP-1, but not NF-κB expression.

Legend for Figure 5.2.5 continued
Hypoxic macrophages were co-incubated with NAC (0.5 or 5 mM) for 30 min, resulting in marked reduction in IL-8 mRNA expression (5.2.6**C** and **D**). Nuclear NF-κB activation was not significantly effected by NAC at this time point (5.2.5**A**), but AP-1 activation was inhibited (5.2.5**B**) The EMSAs shown are representative of n=3 experiments. The IL-8 northern blot and densitometry is one of n=2 experiments.

5.2.6. and 5.2.7. Differential effect of hypoxia on selected chemokine and cytokine mRNA expression.

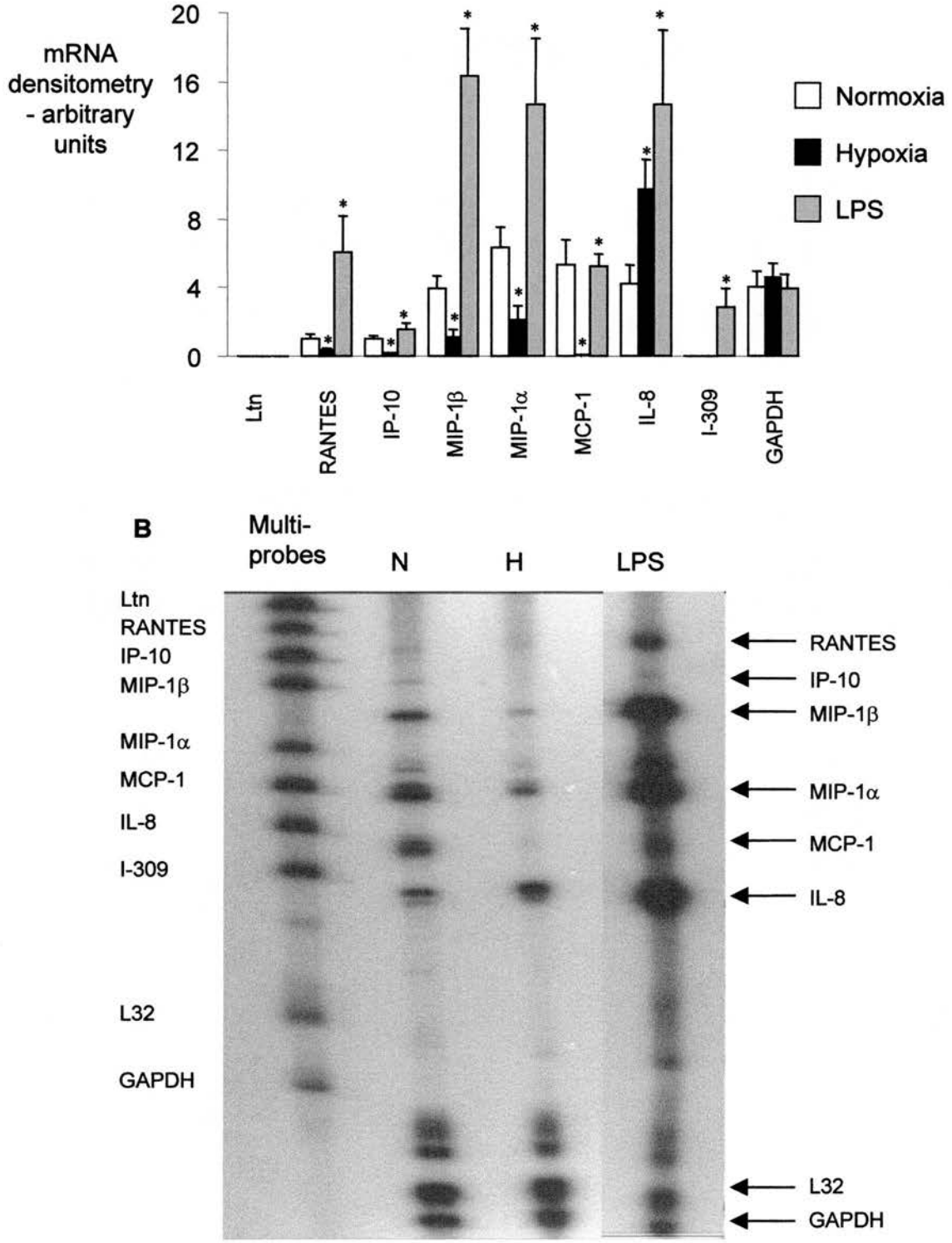
Hypoxia has been shown to regulate a large number of genes (**Table 1.5.6**). Having shown that acute hypoxia increased steady-state expression of IL-8 mRNA in human monocyte-derived macrophages, the effects of hypoxia on a number of other chemokines and cytokines was studied using multi-probe RNase protection assay. Two hours hypoxia significantly increased IL-8 mRNA expression (130±18%, expressed as a percent increase over normoxia. $P<0.01$) and inhibited the expression of RANTES (62±12 %), IP-10 (100 %), MIP-1β (76±9 %), MIP-1α (52±10 %) and MCP-1 (100 %), expressed as percentage inhibition of normoxic controls (all $P<0.02$). In contrast to hypoxia, LPS was able to rapidly upregulate RANTES (590±94 %), 1P-10 (60±38 %), MIP-1β (480±48 %), MIP-1α (396±47 %) and IL-8 (410±60 %) (all $P<0.02$) (**Figure 5.2.6**).
Using a different panel of riboprobes, hypoxia was shown to also have a small but significant inhibitory effect on TNF-α and significantly increased TGF-β1 expression. LPS was a potent inducer of TNF-α and significantly increased TGF-β1 expression. (**Figure 5.2.7.**)

5.2.8 and 5.2.9. Hypoxia increase nuclear AP-1, but not NF-κB levels, using a concensus oligonucleotide probes

The observation that hypoxia increased nuclear AP-1 and C/EBP, but not NF-κB activation was unexpected as most, though not all, inducers of IL-8 gene transcription activate NF-κB (Roebuck, 1999). Furthermore, several of the genes studied with the multiprobe RNase protection assay harbour NF-κB and AP-1 binding sites within the promoter regions (**Figure 5.3.1**). To further explore a potential activation of NF-κB and

AP-1 by hypoxia the experiment was repeated using concensus rather than IL-8-specific oligonucleotide probes. This was particularly of interest for NF-κB, since the IL-8-specific sequence differs by several nucleotides from the concensus sequence. In contrast, the IL-8-specific AP-1 binding harbours the consensus AP-1 footprint sequence (see **Chapter 2.2.7** for sequences). The results for both concensus probes however were similar to that observed with the IL-8 probes; hypoxia had no effect on basal nuclear NF-κB levels (**Figure 5.2.8**), whilst AP-1 nuclear abundance was significantly increased by 15 min of hypoxia (**Figure 5.2.9**).

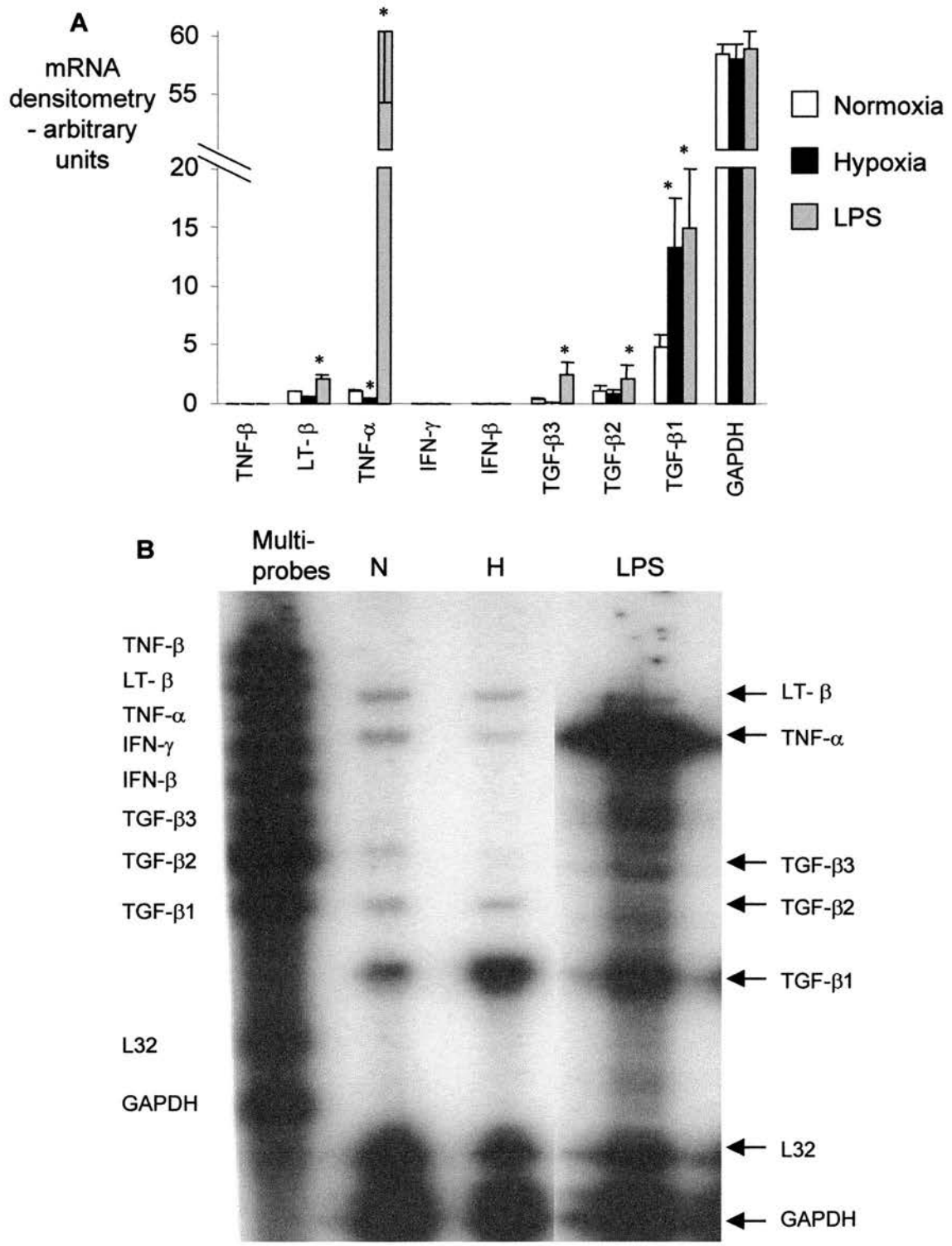
Figure 5.2.6



Legend for Figure 5.2.6. Differential effect of hypoxia on selected chemokines. Macrophages were exposed to normoxia (N), hypoxia (H) or LPS (1 μg/ml) for 2 hours and RNase protection assay performed with a panel of riboprobes. Note that the unprotected riboprobes which make up the ladder are larger and hence do not migrate as

Legend for Figure 5.2.6 continued
far down the gel as the corresponding protected RNA species. IL-8 mRNA is upregulated by hypoxia. Expression of MIP-1β, MIP-1α and MCP-1 was significantly reduced by hypoxia. Expression of RANTES and IP-10 was comparatively low. Prolonged radiographic exposure however confirmed normoxic RANTES and IP-10 expression and reduced expression under hypoxic conditions. In contrast, LPS significantly upregulated the majority of chemokines. Each value in 5.2.6**A** represents mean ± SEM of n=3 separate experiments (*P<0.02). Densitometry for each chemokine was corrected for L32 housekeeping gene expression. A representative assay is shown 5.2.6**B**.

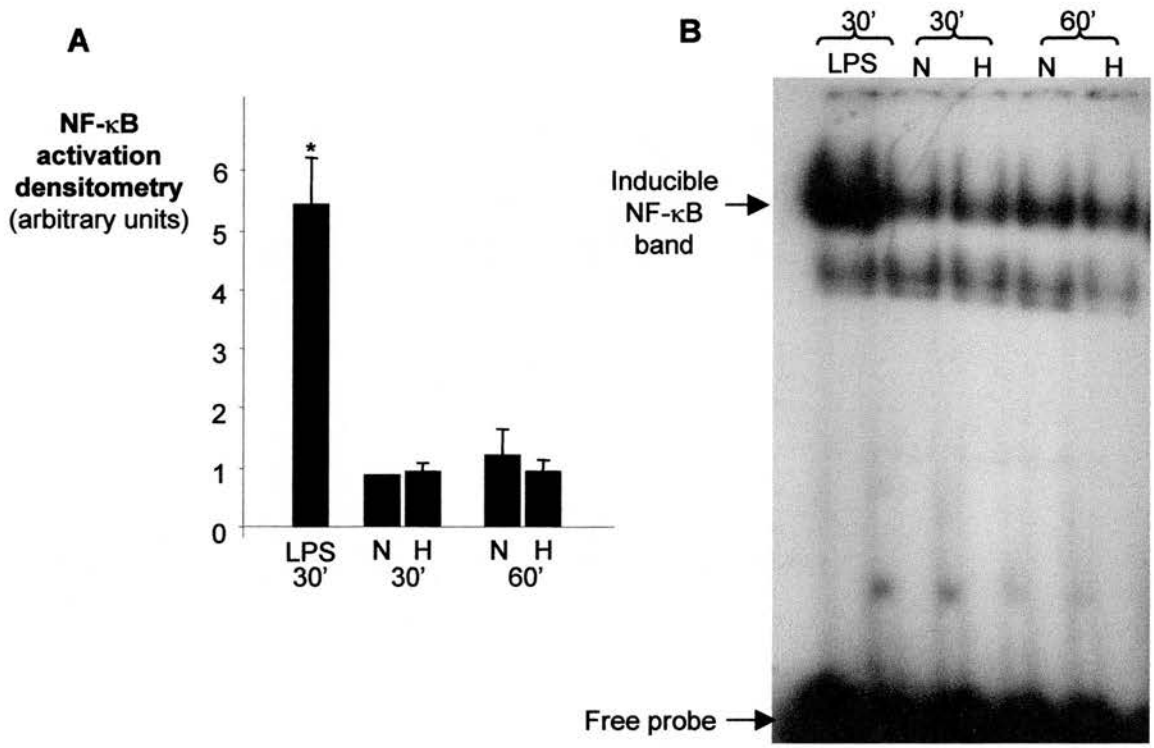
Figure 5.2.7



Legend for Figure 5.2.7. Differential effect of hypoxia on selected cytokines.

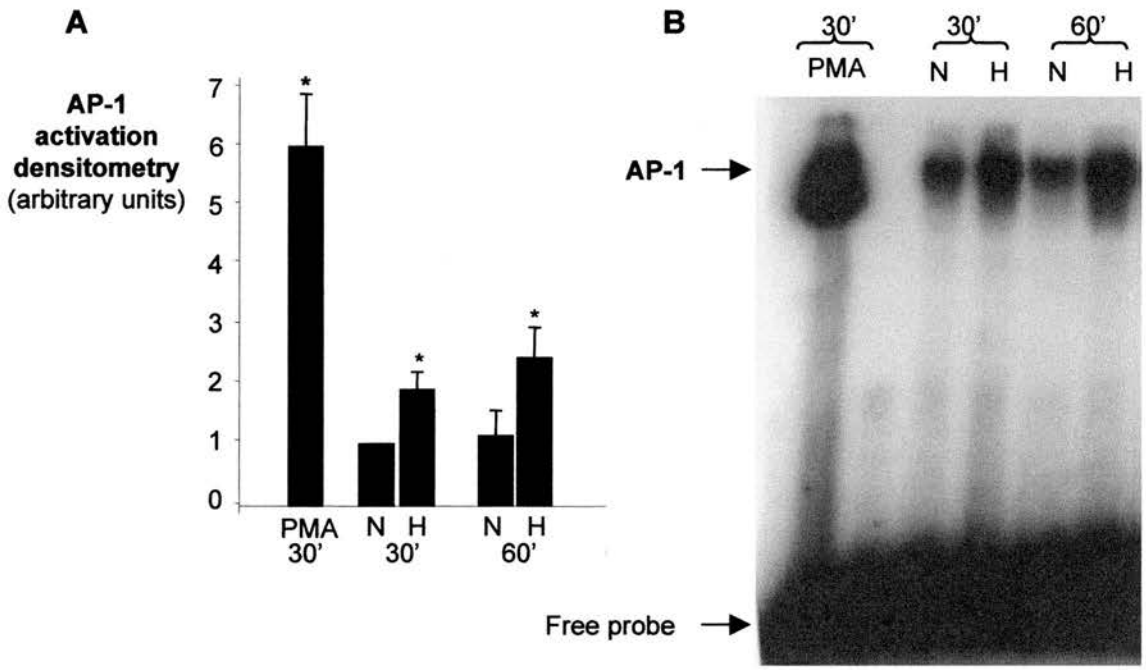
Legend for Figure 5.2.7 continued
Macrophages were exposed to normoxia (N), hypoxia (H) or LPS (1 μg/ml) for 2 hours and RNase protection assay performed with a panel of riboprobes. Compared to normoxia, hypoxia inhibited TNF-α mRNA expression, and significantly increased TGF-β1. LPS significantly increased TNF-α and TGF-β1 expression. TGF-β2 and TGF-β3 were expressed at very low levels in normoxic and hypoxic conditions, although LPS stimulation did increase expression of both cytokines. Expression of IFN-γ and IFN-β were expressed at low or undetectable levels. Each value in 5.2.7**A** represents mean ± SEM of n=3 separate experiments (*P<0.02). Densitometry for each cytokine was corrected for L32 housekeeping gene expression. A representative assay is shown 5.2.7**B**.

Figure 5.2.8



Legend for Figure 5.2.8. Hypoxia does not activate NF-κB – using a concensus oligonucleotide probe.
Macrophages were incubated as previously described for up to 60min and nuclear levels of NF-κB determined by EMSA using a concensus oligonucleotide probe. Hypoxia did not increase NF-κB activity above basal normoxic levels. LPS (1 μg/ml for 30 min) strongly induced NF-κB activation. Each value in Figure 5.2.8**A** represents the mean ± SEM of n=4 separate experiments. (*P<0.01). A representative EMSA is shown (5.2.8**B**), demonstrating that the concensus probe resolved two distinct NF-κB bands, of which the upper (arrowed) is inducible by LPS.

Figure 5.2.9



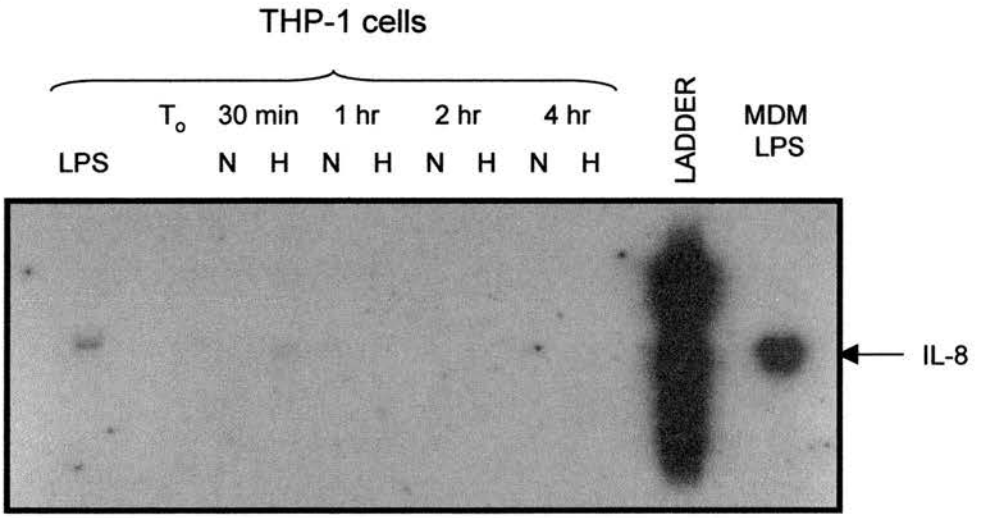
Legend for Figure 5.2.9. Hypoxia does not activate AP-1 – using a consensus oligonucleotide probe. Macrophages were incubated as previously described for up to 60 min and nuclear levels of AP-1 determined by EMSA using a concensus oligonucleotide probe. Hypoxia increase AP-1 activity compared to normoxia by 30 min. PMA (100 ng/ml for 30 min) strongly induced AP-1 activation. Each value in Figure 5.2.9**A** represents the mean ± SEM of n=4 separate experiments. (*P<0.01 compared to normoxic controls). A representative EMSA is shown (5.2.9**B**).

5.2.10. IL-8 expression in hypoxic THP-1 cells

A practical limitation of studying primary monocyte-derived macrophages is the relative paucity of cells compared to a cell line. To establish if the hypoxic upregulation of IL-8 was reproduced in a human monocyte / macrophage cell line, THP-1 cells (see Chapter 2) were exposed to hypoxia and IL-8 mNA expression detected by northern blotting. **Figure 5.2.10** shows that these cells expressed barely detectable levels of IL-8 mRNA under normoxic or hypoxic conditons. Secreted IL-8 in the supernatants was below the level of detection by ELISA. Treatment with LPS induced IL-8 expression, but this was

considerably less than that observed with monocyte-derived macrophages treated with LPS.

Figure 5.2.10

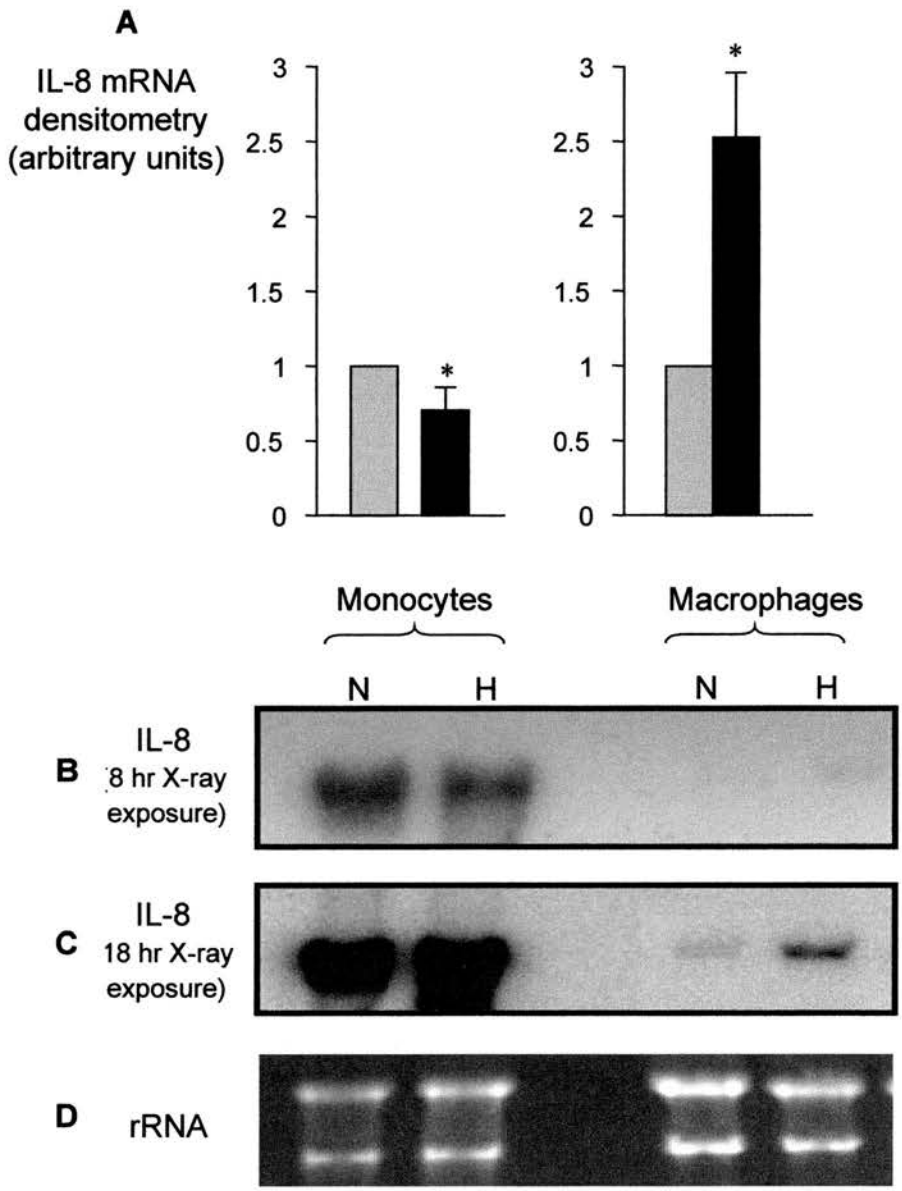


Legend for Figure 5.2.10. IL-8 mRNA expression in THP-1 cells.
IL-8 mRNA expression in normoxic and hypoxic THP-1 cells cultured for up to 4 hours was barely detectable by northern blotting. Treatment with LPS (4 hours) generated detectable IL-8 expression, but this was considerably less than that observed in primary monocyte-derived macrophages (MDM) treated with LPS (4 hours). The blot shown represents one of n=2 separate experiments.

5.2.11. IL-8 expression in human monocytes

Finally, the effect of acute hypoxia in primary human monocytes was studied. It was anticipated the hypoxic IL-8 response in monocytes would be similar to that observed in its derivative cell, the mature monocyte-derived macrophage. However, whereas IL-8 gene expression increased in response to hypoxia in the monocyte-derived macrophage (cultured over 5 days), in the monocyte (cultured over 1 day) hypoxia resulted in a small but significant inhibition in steady-state IL-8 mRNA expression (**Figure 5.2.11.**)

Figure 5.2.11



Legend for Figure 5.2.11. Hypoxia inhibits basal IL-8 mRNA expression in cultured human monocytes.

Human peripheral blood monocytes (aged 18 hr) and macrophages (aged 5 days), both from the same donor, were cultured in normoxic (N) or hypoxic (H) conditions for 4 hours. Unstimulated monocytes in the culture conditions used expressed significantly more IL-8 than the matured macrophage, hence a relatively short X-ray exposure time (8 hr) was required to allow densitometry measurement (5.2.11**B**). A more prolonged exposure (18 hr) was required to allow macrophage IL-8 quantification (5.2.11**C**). Hypoxia resulted in a small but significant reduction in basal IL-8 secretion from monocytes, but increased IL-8 mRNA expression in macrophages. The values in 5.2.11.**A** represent the mean and SEM from 3 separate donor experiments. IL-8 densitometry was corrected for 18s house-keeping expression, or ethidium bromide stained ribosomal RNA (rRNA) as shown in 5.2.11.**D.**

5.3 DISCUSSION

In this chapter, it was shown that hypoxia increased IL-8 mRNA expression in human monocyte-derived macrophages. This increase occurred within 30 min of hypxoic exposure and in association with raised nuclear levels of AP-1 and C/EBP-β, but not NF-κB. N-acetylcysteine was shown to attenuate hypoxic IL-8 upregulation in association with reduced nuclear AP-1 expression. Hypoxia was found to differentially influence expression of a range of chemokines and cytokines in macrophages. The pattern of transcription factor activation and the chemokine and cytokine expression induced by hypoxia was different to that induced by endotoxin.

Hypoxia was found to rapidly increase steady-state IL-8 mRNA expression by 30 min compared to normoxic controls and T_0 cells. This suggests that hypoxia rapidly increases IL-8 mRNA expression, at least in part through increased transcription. However, a nuclear runoff transcription assay, currently the most sensitive method for measuring specific gene transcription (Greenberg and Bender, 1997), was not performed. It is possible that hypoxia also increases IL-8 mRNA stability. Kunz et al., (1999) have recently demonstrated increase IL-8 transcription and enhanced mRNA stability in a melanoma cell line cultured in anoxic conditions. Whether this also applies to macrophages requires further study.

In Chapter 3, it was demonstrated that IL-8 protein expression from monocyte-derived macrophages was significantly raised at 2 hours, but not at other time-points (**Figure 3.2.5**). Steady-state hypoxia-induced IL-8 mRNA expression was found to be raised up to the 4 hour time point studied. Macrophages did not have detectable levels of stored IL-8 under hypoxic or normoxic conditions, suggesting inhibition at the level of protein translation. Hence hypoxia appears to increase IL-8 mRNA expression rapidly and persistently over 4 hours without parallel increase in protein expression. Studies in epithelial cells treated with respiratory syncytial virus (RSV) or influenza A virus (Choi and Jacoby, 1992; Fiedler et al., 1996) and in keratinocytes treated with chemical 'sensitizers' (benzene compounds) (Mohamedzadeh et al., 1994), have demonstrated a

similar phenomenon, with significant IL-8 gene transcription but comparatively little or no protein expression. In this context, it has been postulated that the upregulation of IL-8 mRNA may represent an initial response, 'arming' the cell in preparation for a subsequent potentially injurious stimulus such as secondary bacterial infection or further chemical insult. This hypothesis may apply to the hypoxic upregulation of IL-8 mRNA in macrophages, in which a subsequent insult may be reoxygenation or endotoxin.

Increased IL-8 gene expression in macrophages by hypoxia was rapid, occurring within 30 minutes of exposure. Hypoxia has been reported to increase IL-8 expression in several other cell types, but in none has the increase been reported to occur as rapidly as in monocyte-derived macrophages reported in the presented study. In primary human fibroblasts and pulmonary vascular smooth muscle cells, exposure to 3% environmental oxygen resulted in increased IL-8 mRNA expression by 8 hours exposure, and protein by 48 hours exposure (Tamm et al., 1998). Bovine retinal glial cells in an anaerobic environment have been shown to increase IL-8 mRNA expression by 3 hours (Yoshida et al., 1998) but earlier time-points were not reported in this study. Several tumour cell-lines, including human glioblastoma (Desbaillets et al., 1997) pancreatic adenocarcinoma (Shi et al., 1999), ovarian (Xu et al., 1999) and malignant melanoma (Kunz et al., 1999) have been shown to upregulate IL-8 in response to hypoxia or anoxia over several hours or days.

It is possible that the monocyte-derived macrophage is a comparatively rapid hypoxic responder. However, apparent differences in response-rate to hypoxia may be entirely attributable to differences in experimental protocol between studies. In the studies of different cell lines described, medium was not pre-incubated in the hypoxic environment and the PO_2 of the medium not directly measured. As discussed in Chapter 3, both factors may be important in determining the hypoxic responses. Karakurum et al., (1994) has shown increased IL-8 mRNA expression in human umbilical endothelial cells (HUVECs) exposed to 6 hours hypoxia. The PO_2 of the medium was reported to be ~ 2 KPa, although the method used to measure oxygen tension, or the time at which this was measured, was not described.

The 5'-regulatory region of the IL-8 gene contains several potential transcription regulatory elements (**Figure 5.1.1**). Based principally upon studies of mutated and restricted reporter-linked IL-8 gene promoter constructs, three transcription factors in particular, NF-κB, AP-1 and C/EBP-β (NF-IL-6) have been implicated in regulating IL-8 gene transcription (Mukaida N et al., 1994; Roebuck et al., 1999). The relative importance of these transcription factors appears to be dependent upon both the cell type and the stimulus. In this study, it has been demonstrated that hypoxia rapidly increased nuclear abundance of both AP-1 and C/EBP-β, within 15 min of exposure. In contrast, there was no increase in nuclear NF-κB levels compared to normoxic controls at the early time-points investigated. This pattern of activation differs from that seen with LPS, which strongly induced NF-κB activation in particular.

These data pertaining to transcription factors are based upon EMSA analysis. This technique assays nuclear levels or abundance of a given transcription factor. This does not necessarily provide a measure of transcription factor 'activation' (the signalling events that generate the final nuclear-translocating transcription factor complex) or functionality (the subsequent induction of gene transcription). However, based upon current understanding of AP-1, NF-κB and C/EBP-β regulation and their role in IL-8 transcription, discussed herein, the presence of these transcription factors within the nucleus implies activation and this term is used as such.

A series of promoter mutation studies indicate that the NF-κB binding site is the predominant *cis*-acting element in the IL-8 gene expression (reviewed in Roebuck, 1999). Hence, IL-8 transcription induced by TNF-α (Lakshminarayanan et al., 1998), respiratory syncytial virus (Mastronarde et al., 1998) and rhinovirus (Zhu Z et al., 1997) in A549 cells, is critically dependent upon the presence of the NF-κB-binding element. Similar observations have been made in IL-β stimulated CaCO (colonic cancer) cells (Wu GD et al., 1997) and HUVECs treated with heavy metal ions (Wagner et al., 1998). However, in all cases, maximal IL-8 gene induction was only achieved in the presence of non-mutated AP-1 and / or C/EBP-β binding elements. Furthermore, Roebuck and colleagues have recently reported NF-κB - independent, AP-1 - dependent IL-8 gene induction in A549 cells treated with H_2O_2 (Lakshminarayanan et al., 1998; Roebuck et al., 1999).

The NF-κB family of transcription factors comprise a group of structurally related Rel proteins. These include Rel-A (p65), Rel-B, c-Rel, NF-κB-1 (p50) and NF-κB-2 (p52). The NF-κB complex is composed of homodimers or heterodimers of the Rel proteins (Perkins et al., 1992). In 'resting' cells, latent NF-κB is sequestered in the cytoplasm, complexed to IκB (a family of cytoplasmic retention proteins) (May and Ghosh, 1998). Upon cell stimulation, protein kinase signalling cascades result in IκB-α phosphorylation, an event that induces ubiquitination of the cytoplasmic-retaining protein and permits NF-κB translocation to the nucleus (Ghosh and Baltimore, 1990). Raised nuclear NF-κB levels therefore infer cytoplasmic transcription factor activation. The signalling events leading to IκB-α phosphorylation have recently been reviewed (Silverman and Maniatis, 2001). In the NF-κB EMSA study described in this chapter (**Figure 5.2.2**), normoxic monocyte-derived macrophages were found to have basal levels of nuclear NF-κB (and indeed AP-1 and C/EBP-β). Most studies of NF-κB activity in monocytes and monocyte-derived macrophages have revealed constitutive nuclear NF-κB expression (Xu XP et al., 1999; Blanco-Colio et al., 2000). The extent to which serum factors are responsible for this is not certain, though Frankenberger et al., (1994) have also demonstrated basal nuclear NF-κB expression in primary human monocytes in serum-free conditions. In the studies described in this thesis, monocytes in 10% autologous serum to derive macrophages. Although serum-free culture protocols have been described for monocyte culture, in general the yield of mature macrophages is comparatively poor (Dr Ian Dransfield, personal communication). Actual macrophage experiments were performed in 2% autologous serum and this partial 'serum withdrawal' may be in part responsible for constitutive transcription factor activation.

The IL-8 NF-κB binding site appears to bind a restricted subset of Rel-family proteins. Dimers consisting of p65, p50 and c-Rel complexes appear to constitute the IL-8 promoter-binding NF-κB complex in most studies (Kunsch et al., 1994; Roebuck, 1999). The supershift EMSA study in **Figure 5.2.2** was consistent with the conclusion that the LPS-induced NF-κB species detected in monocyte-derived macrophages comprised both p50 and p65 subunits. The precise dimeric species can not be identified from this study

and other Rel protein may be harboured in the DNA-binding NF-κB complex. Acute hypoxia was found not to influence nuclear NF-κB abundance. At least two previous studies have implicated NF-κB in the hypoxic upregulation of IL-8. In HUVECs, Karakurum et al., (1994) demonstrated an enhanced NF-κB - IL-8 promoter binding band on EMSA, induced by 6 hours hypoxia, that did not supershift with p50 or p65 antibody. Yoshida et al., (1998) also report increased nuclear NF-κB levels in bovine retinal glial cells by 1 hour anoxia (without pre-incubation) in association with p65 nuclear translocation. The data presented in this thesis do not imply that NF-κB is superfluous to hypoxia-induced IL-8 transcription in monocyte-derived macrophages. Since cells showed basal nuclear NF-κB expression, it is possible that constitutive activation of NF-κB is necessary for subsequent hypoxic upregulation.

Since the IL-8 promoter appears to only bind a subset of NF-κB proteins, EMSA was also performed with a consensus NF-κB oligonucleotide harbouring the 'classical' NF-κB binding element 5'-GGGGACTTTCC-3' (**Table 2.2.7**). This again showed acute hypoxia to have no significant effect on nuclear NF-κB abundance. In contrast, using similar consensus NF-κB probes but more prolonged hypoxia (2 hours), Lepper-Woodford and Detmer, (1999) demonstrated enhanced nuclear NF-κB levels in rat alveolar macrophages and Chandel et al., (2000) have shown increased nuclear NF-κB in a murine macrophage cell line. The different results between these two studies and the current study in human monocyte-derived macrophages may reflect the different cell phenotypes or the relatively short exposure (30 min) employed in the studies presented herein.

Several studies, mainly based on evidence from reporter-linked data, have implicated AP-1 in IL-8 gene regulation (Roger et al., 1998; Lakshminarayanan et al., 1998; Mori et al., 1998). AP-1 is dimeric complex composed of proteins of the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra 1 and Fra 2) or ATF (activating transcription factor) family (Karin et al., 1997). Most frequently, the AP-1 complex comprises Jun-Jun homodimers or Jun-Fos heterodimers (Smeal et al., 1989). The cytosolic subunits join via leucine zipper domains and this dimerisation is essential for nuclear translocation and for binding of the

functional transcription factor to DNA (Abate et al., 1990). In this respect, measuring nuclear abundance provides an indication of AP-1 activation.

Nuclear levels of AP-1 were raised within 15 min of hypoxic exposure in macrophages. Rapid nuclear AP-1 accumulation following hypoxia has previously been demonstrated in other cell types, including HT29 colonic cancer cells (Yao et al., 1994), HUVECs (Bandyopadhyay et al., 1995) and HeLa cells (Bandyopadhyay et al., 1995; Rupec and Baeuerle, 1995). In the AP-1 supershift study described in this chapter, IL-8 DNA binding AP-1 species was shown to contain c-Jun protein, but no supershift was observed with c-Fos antibody. In the absence of further superhift studies with alternative AP-1 subunit antibodies, the precise components of the detected AP-1 species cannot be determined. There have been relatively few studies which have sought to identify the constituents of AP-1 protein binding to the IL-8 promoter (Roebuck et al., 1999). In A549 cells (a type 2 alveolar epithelial cell line), supershift analysis of H_2O_2 or TNF-α - induced nuclear AP-1 revealed c-Fos and JunD subunits, but not c-Jun (Lakshminarayanan et al., 1998). Jurkat cells stimulated with PMA expressed increased IL-8 gene expression in association with increased nuclear c-Fos / JunD complex accumulation (Mukaida et al., 1990).

In contrast to NF-κB, the IL-8 AP-1 binding element is homologous with the 'classical' AP-1 binding motif (5'-TGA($^{C}/_{G}$)TCA-3', **Table 2.2.7**). Results from EMSA studies performed with a consensus AP-1 oligonucleotide were identical to those with the IL-8 AP-1 probe, revealing increased nuclear AP-1 abundance following acute hypoxia and PMA.

The basic-leucine zipper dimeric protein C/EBP-β was originally identified as a critical transcription factor for IL-6 transcription (Poli et al., 1990). Within the IL-8 promoter, C/EBP-β has been reported to co-operate with NF-κB, such that binding of both transcription factors increases gene transcription synergistically (Kunsch et al., 1994). In the absence of NF-κB binding, C/EBP-β binds weakly to the IL-8 promoter. This cooperativity has been described in A549 cells stimulated with RSV (Mastronarde et al., 1998), ozone (Jaspers et al., 1997) and asbestos fibres (Simeonova and Luster, 1996) and

in PMA-treated Jurkat cells (Kunsch et al., 1994). The EMSA studies in this thesis reveal enhanced nuclear C/EBP-β levels following acute hypoxia. Enhanced C/EBP-β activation by hypoxia has also previously been reported in cultured endothelial cells (Yan et al., 1995). In transgenic mice harbouring the C/EBP-β binding element ligated to a *lacZ* reporter, hypoxia was found to increase β-galactosidase expression in the vasculature of lungs, heart and kidneys (Yan et al., 1997).

A proposed model for the regulation of NF-κB, AP-1 and more recently C/EBP-β is through cellular redox status and reactive oxygen species (ROS) (Li and Karin, 1999 in FASEB; Gomez-del-Arco et al., 1996; Kastan, 1997; Rahman and MacNee, 2000). Reactive oxygen species are implicated in the two redox models discussed in **Chapter 1.5.5.** (**Figure 1.5.5**). In the NAD(P)H model, ROS are generated by a membrane-bound NAD(P)H oxidase in direct proportion to environmental oxygen. Hypoxia would therefore result in decreased ROS. In the mitochondrial electron transport chain (ETC) model, hypoxia inhibits the activity of cytochrome oxidase (cyt aa_3), the terminal electron receiver, hence increasing downstream generation of ROS. In both cases, the change in intracellular ROS would be central to subsequent signalling events.

In the presented data, NAC was found to inhibit hypoxia-induced IL-8 gene transcription, in association with reduced nuclear AP-1 accumulation, but with no effect on basal NF-κB activity. NAC functions primarily as an anti-oxidant through two distinct mechanisms; the direct scavenging of ROS and reduction of sulphydryl groups, and also indirectly as a substrate for the biosynthesis of the cellular redox regulator glutathione (L-glutamyl-L-cysteinylglycine, GSH) (Gillissen and Nowak, 1998; Kelly, 1998; Rahman and MacNee, 2000). The attenuation of hypoxia-inducible IL-8 with NAC would support a model in which hypoxia increased ROS, signalling AP-1 activation and gene transcription in monocyte derived macrophages. Since basal expression of NF-κB was unchanged with hypoxia or NAC treatment, the relevance of constitutive NF-κB activation in subsequent hypoxic activation is unresolved.

The precise regulatory site for hypoxia in the AP-1 activation pathway however is elusive. AP-1 may be regulated at the transcriptional and post-transcriptional level. At the

transcriptional level, the immediate early genes *c-jun* and *c-fos* are upregulated within a few minutes of hypoxic exposure in a range of cells and tissues (Webster et al., 1993; Ausserer et al., 1994). In pulmonary artery fibroblasts, hypoxia activates p38 mitogen-activated protein kinase (MAPK) activity and downstream c-Jun expression (Scott et al., 1998). Pharmacological reduction in intracellular ROS also induces (MAPK) activation, downstream *c-fos* and *c-jun* induction and c-Fos and c-Jun protein expression (Muller et al., 1997; Lo, 1996). Post-transcriptionally, reduction and phosphorylation events regulate transcription factor-DNA binding. Both Fos and Jun proteins bear a redox sensitive conserved cysteine residue, reduction of which is necessary to allow electrostatic interaction between protein and DNA (Abate et al., 1990). This would however infer that AP-1-mediated transactivation would increase in the presence of a reducing agent such as NAC. This is indeed the case in HeLa cells for example, in which unstimulated and PMA-stimulated AP-1 binding and transactivation is increased in the presence of NAC (Bergelson et al., 1994; Meyer et al., 1993). Finally, phosphorylation of two serine residues in the transactivation domain of c-Jun is also required for transcriptional activation (Karin, 1995). Increased ROS following treatment with H_2O_2 has recently been shown to activate JNK (Jun N-terminal kinase) through allowing its dissociation from an inhibitory protein termed GSTp (glutathione S-transferase). Activation of JNK results in c-Jun phosphorylation and AP-1 transactivation, a process inhibited by NAC (Adler et al., 1999). Hence, this model may also be consistent with the hypothesis that hypoxia generates ROS and AP-1 activation.

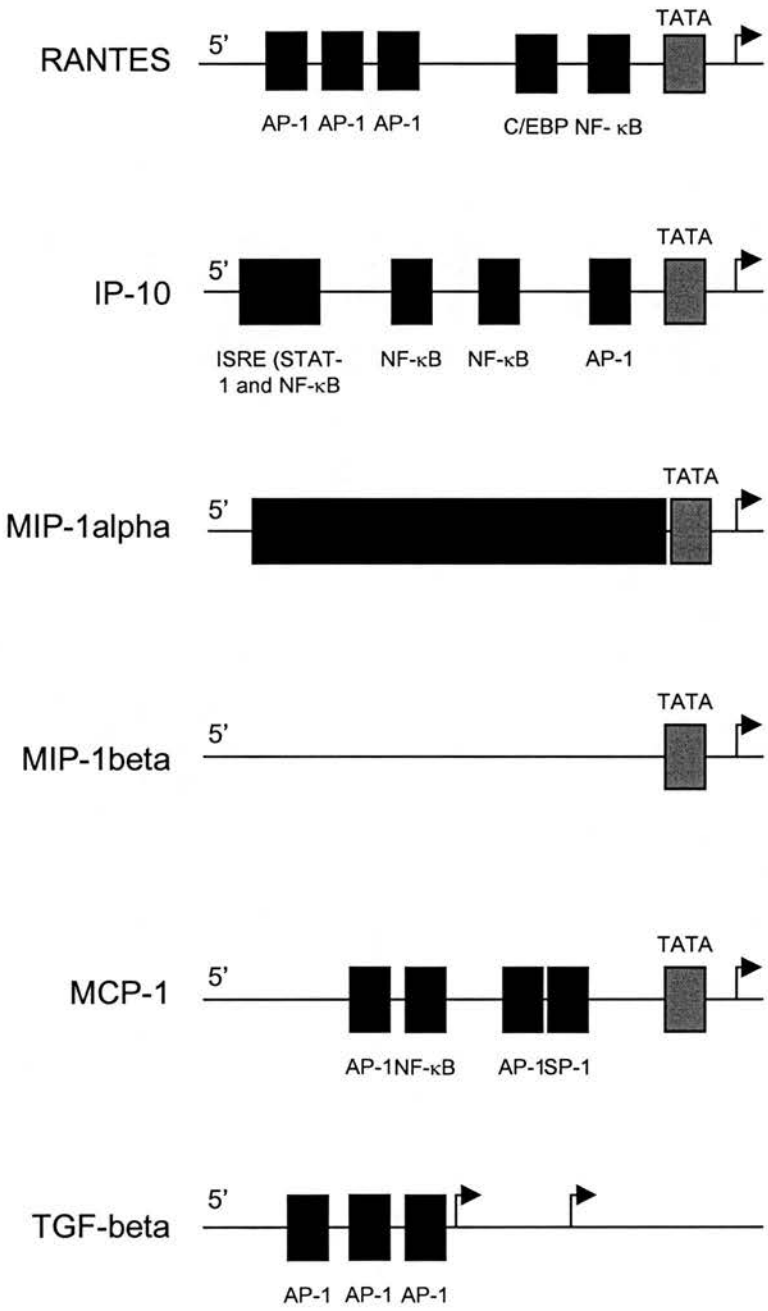
Several studies have shown that anti-oxidants inhibit NF-κB activation and the downstream expression of NF-κB responsive genes (reviewed in Li and Karin, 1997). The antioxidant effect, as with AP-1, appears to be highly cell- and stimulus-specific (Schreck et al., 1991; Brennan and O'Neill, 1995; Li and Karin, 1999; Rahman and MacNee, 2000). Two recent studies that have demonstrated NF-κB activation in response to hypoxia have also reported attenuation following antioxidant treatment with NAC (Chandel et al., 2000; Haddad et al., 2000), implicating increased ROS in hypoxic signalling. As with AP-1, the regulatory site within the NF-κB activation pathway is unknown. Oxidative stress is associated with IκB-α phosphorylation, an event that is inhibited by antioxidants including NAC in endothelial cells (Cho et al., 1998) but not

HeLa cells (Li and Karin, 1999). Although 'classical' IκB-α phosphorylation occurs at two conserved serine residues, there is evidence that hypoxia may induced IκB-α phosphorylation at tyrosine residues (Koong et al., 1994).

The finding that acute hypoxia increased AP-1 and C/EBP-β, but not NF-κB, nuclear expression may have important implications for the selectivity of the hypoxic response in macrophages. Both putative and functional binding sites for the three transcription factors studied are present in a large number of gene promoters. The observation that the pattern of activation with concensus probes was identical to that with IL-8-promoter probes would suggest the potential for hypoxia to regulate many genes. The presented data (Figures **5.2.7** and **5.2.8**) shows that, in contrast to IL-8, basal steady-state mRNA expression of several chemokines and cytokines, were clearly down-regulated by 2 hrs hypoxia. The most striking effects were the reduction in basal expression of the CC chemokines MIP-1α, MIP-1β and MCP-1. Low level expression of IP-10, a non-ELR harbouring CXC chemokine, was attenuated. Of the cytokines studied, basal TNF-α expression was inhibited and only TGF-β1 expression was significantly increased by hypoxia. This pattern of expression contrasted starkly with that observed with LPS.

The published promoter regions of a selection of the chemokines and cytokines studied is shown in **Figure 5.3.2**. It is apparent that AP-1 and NF-κB binding sites are common to several of the chemokine genes, of which the most extensively studied is MCP-1. Binding of Sp-1 to the proximal MCP-1 promoter is known to be critical for assembly of basal transcriptional machinery following TNF-α stimulation (Ping et al., 2000). However, both NF-κB and AP-1 sites are required for maximal transcriptional upregulation of MCP-1 in response to most inflammatory stimuli including TNFα, IL-β, viral proteins and H_2O_2 (Martin et al., 1997; Lim and Garzino-Demo, 2000; Goebeler et al., 2001; Roebuck et al., 1999; Wung et al., 1997). Therefore, it appears that the transcriptional regulation of MCP-1 is not unlike that of IL-8. There are several potential explanations that may explain the observation that in human macrophages, hypoxia increased steady-state IL-8 mRNA, in association with increased nuclear AP-1 levels and maintained basal NF-κB activation, yet abolished MCP-1 expression.

Figure 5.3.1



Legend for Figure 5.3.1. Schematic representation of the promoter regions of selected chemokines and TGF-β harbouring AP-1, NF-κB and C/EBP binding elements. GRE = glucucorticoid responsive element. IRSE = interferon stimulated response element.

It is possible that the effect of hypoxia, at least in part, induces differential stability of mRNA species. This aspect of mRNA expression was not studied. Hypoxia is known to increase mRNA stability through induction of cytoplasmic hypoxia-inducible proteins (HIP) that bind to the AU-rich 3'untranslated region (UTR) of the VEGF, tyrosine hydroxylase and erythropoeitin mRNA species (Shih and Claffey, 1998; Lewis et al., 1999). There are no previous reports of hypoxia *reducing* mRNA stability however. Furthermore, the acute effect (2 hours) may be too rapid to implicate stability as a factor in mRNA expression.

Another possibility is that the differential regulation is AP-1 – mediated, with the transcription factor inducing repression in some genes, such as MCP-1 and activation in others, such as IL-8 and TGF-β. Hypoxia upregulation of TGF-β1 has previously been reported in proximal tubular epithelial cells (Orphanides et al., 1997), retinal cells (Khaliq et al., 1995, Behzadian et al., 1998) and a choreocarcinoma cell line (Gleadle et al., 1995). Transcriptional control of TGF-β1 appears to be largely AP-1 mediated (Figure 5.3.2), and Helfman and Falanga (1993) have demonstrated requirement of AP-1 binding sites for maximal hypoxic gene induction. With regard to MCP-1 transcription, a functional repressor transcription factor complex has been reported to regulate MCP-1 gene expression (Jain-Vora et al., 1997; Sridhar et al., 1999). However, there are no previous reports of AP-1 acting as a gene silencer and indeed within the MCP-1 promoter, AP-1 has been shown to be critical for oxidant (H_2O_2) induced gene transcription (Wung et al., 1997). Furthermore, several of the genes studied do not harbour AP-1 regulatory elements within the known promoter regions. Hence, it is unlikely that AP-1 acts a central hypoxic regulator within this panel of genes.

This raises the possibility that an alternative transcription factor or factors are responsible for the differential regulation observed. The most obvious candidate is HIF-1, which has been termed a master regulator of hypoxic gene responses (Semenza et al., 1999). The role of HIF-1 in IL-8 gene transcription is explored further in **Chapter 6**. However, there is evidence that HIF-1 may play a role in MCP-1 regulation. Negus et al., (1998) have also demonstrated inhibition of MCP-1 mRNA expression by 2 hours in an ovarian cell line stimulated with TNF-α and cultured in 0% oxygen. Consistent with the observations

presented in this chapter, hypoxic inhibition occurred without influencing NF-κB activity as measured by EMSA. A similar inhibition of MCP-1 mRNA was observed with CoCl and DFO, pharmacological mimickers of hypoxia. This provides indirect evidence for a HIF-1 mediated effect. Against this perhaps is the observation that although over 30 individual genes have been shown to be clearly HIF-1 mediated, very few are repressed through a HIF-1 mechanism (Chapter 1.5; Narravula and Colgan, 2001).

Lastly, it is possible that hypoxia influences an aspect of basal transcriptional machinery or a higher order of gene regulation such as chromatin modelling. It has been proposed that the tight interaction between the negatively charged DNA backbone and core histones creates a 'chromatin barrier', impeding the access of regulatory transcription factors to target DNA (Struhl, 1998). This mechanism has been implicated in gene silencing or repression. Acetylation of specific lysine residues, on N-terminal histone tails, through increased activity of specific histone acetylation transferases (HATs) or reduced activity of specific histone deactylases (HDACs), is believed to loosen the DNA-histone interaction and expose previously 'hidden' DNA regions to regulatory transcription factors. This model is attractive in hypoxic gene regulation since it may account for differential regulation of multiple genes simultaneously (Van Lint et al., 1996; Della Ragione et al., 2001). That chemokines, and more specifically MCP-1 and IL-8, may be differentially regulated through histone acetylation / deacetylation, has been demonstrated recently by Fusunyan et al., (1999) and Wen and Wu (2001).

The biological relevance of this differential gene response to acute hypoxia in macrophages is to some extent speculative. The original rationale for studying acute hypoxic regulation was as a mechanism for rapid IL-8 generation in the lungs of patients at-risk of ARDS. Since only IL-8 and not a range of other inflammatory mediators was associated with progression to ARDS, a differential stimulus such as hypoxia may be more relevant than a pan-inflammatory stimulus such as endotoxin. However, the differential effect of hypoxia has not been studied in alveolar macrophages and may not necessarily be relevant in this setting. Monocyte-derived macrophages serve as a reasonable model of tissue macrophages elsewhere and hypoxia is a relevant stimulus in injured, inflamed or malignant tissue (Lewis et al., 1999). Interleukin-8 and TGF-β are

angiogenic, in contrast to IP-10 which is angiostatic, and early induction of IL-8 and TGF-β suggests that tissue repair mechanisms may be initiated even in the acute phase of injury. The clear difference between the pattern of transcription factor activation and chemokine and cytokine generation between hypoxia and LPS is of interest. Macrophages must adapt and respond to a changing environment as they migrate to sites of tissue injury and inflammation. Differential expression of genes to hypoxia and endotoxin may represent a switch from initiating wound healing to responding to infection. It is likely ofcourse that both infection and hypoxia may co-exist, for example in sepsis and the combined effect needs to be investigated further in *in vitro* studies. Therapeutically, it may be possible to attenuate one response, whilst maintaining the other. For example, it may be advantageous to inhibit the comparatively large IL-8 response to LPS if accumulating neutrophils cause overwhelming tissue injury, but to maintain the relatively low-level hypoxic response to aid wound healing.

Table 5.3.1

Transcription Factor	Gene	Reference
NF-κB	IL-8*	Karakurum et al., 1994; Yoshida et al., 1998
	Cyclo-oxygenase-2	Schmedtje et al., 1997
	TNF-α	Leeper-Woodford and Detmer, 1999, Chandel et al., 2000
AP-1	Tyrosine Hydroxylase	Czyzyk-Krzeska et al., 1994a; Czyzyk-Krzeska et al., 1994b; Norris and Millhorn, 1995 Mishra et al., 1998
	Endothelin-1**	Yamashita et al., 2001
	IL-8*	Shi et al., 1999, Xu et al., 1999
	Basic fibroblast growth factor	Le et al., 1999
	TGF-β1	Helfman and Felanga, 1993
C/EBPβ (NF-IL-6)	IL-6	Yan et al., 1996; Yan 1997

Table 5.3.1. Hypoxia-induced genes in which NF-κB, AP-1 and C/EBP-β have been implicated

*Relative importance of NF-κB and AP-1 regulation may be cell-specifc. The studies from Shi et al., 1999 and Xu et al., 1999, using IL-8 reporter constructs, report that both AP-1 and NF-κB sites are necessary for hypoxic induction in pancreatic adenocarcinoma and ovarian carcinoma cells

**Cooperative induction through HIF-1, AP-1, GATA-2 and CAAT-binding factor (NF-1).

CHAPTER 6

STUDIES IN THE ROLE OF HIF-1 IN THE HYPOXIC REGULATION OF INTERLEUKIN-8

6.1 INTRODUCTION

In Chapter 5, NF-κB, AP-1 and C/EBP-β, transcription factors known to be involved in IL-8 gene regulation, were studied in hypoxic macrophages. Hypoxia-inducible factor-1 (HIF-1) is known to be important in the regulation of a number of hypoxia-regulated genes (**Table 1.5.6**). The role of HIF-1 in the hypoxic regulation of IL-8 is unknown. A computer-assisted search (TFSEARCH version 1.3, Yutaki Akiyama, Kyoto University, Japan) for the HIF-binding core sequence 5'-CGTG-3' in the published 1.5Kb IL-8 promoter region (Accession no. M28130; Mukaida et al., 1989) revealed a single putative binding site on the antisense strand located –2 to –6 bp (3'-TCGTGA-5'), immediately upstream from the transcription initiation site, but down-stream of the TATA box (**Figure 5.1.1**). Although functional HIF-1 sites within antisense strands have been reported in other genes (**Table 1.5.6**), a location downstream of the TATA box would be unusual for a regulatory transcription factor element. Furthermore, as the number genes found to be HIF-1 regulated increases, the core binding sequence for the protein has been refined to 5'-RCGTG-3', where R represents a purine (Semenza, 2000). Although these factors suggest the putative IL-8 HIF-1-binding site is unlikely to be functional, it is recognised that HIF-1 may exert its effect at enhancer sites, potentially several Kb away from the TATA box. Recently, novel regulatory *cis*-acting elements located >6.5 kB upstream and / or 3.7 kB downstream of the IL-8 TATA box have been described (Wen and Wu et al. 2001)

The primary aim in this chapter was to explore the role of HIF-1 in hypoxia-induced IL-8 transcription.

6.2 RESULTS

Three approaches were employed to study the potential role of HIF-1 in hypoxic regulation of IL-8; pharmacological studies using mimickers of hypoxia, transfection studies in macrophages using a reporter (CAT)-linked IL-8 promoter and studies in HIF-1-deficient cells.

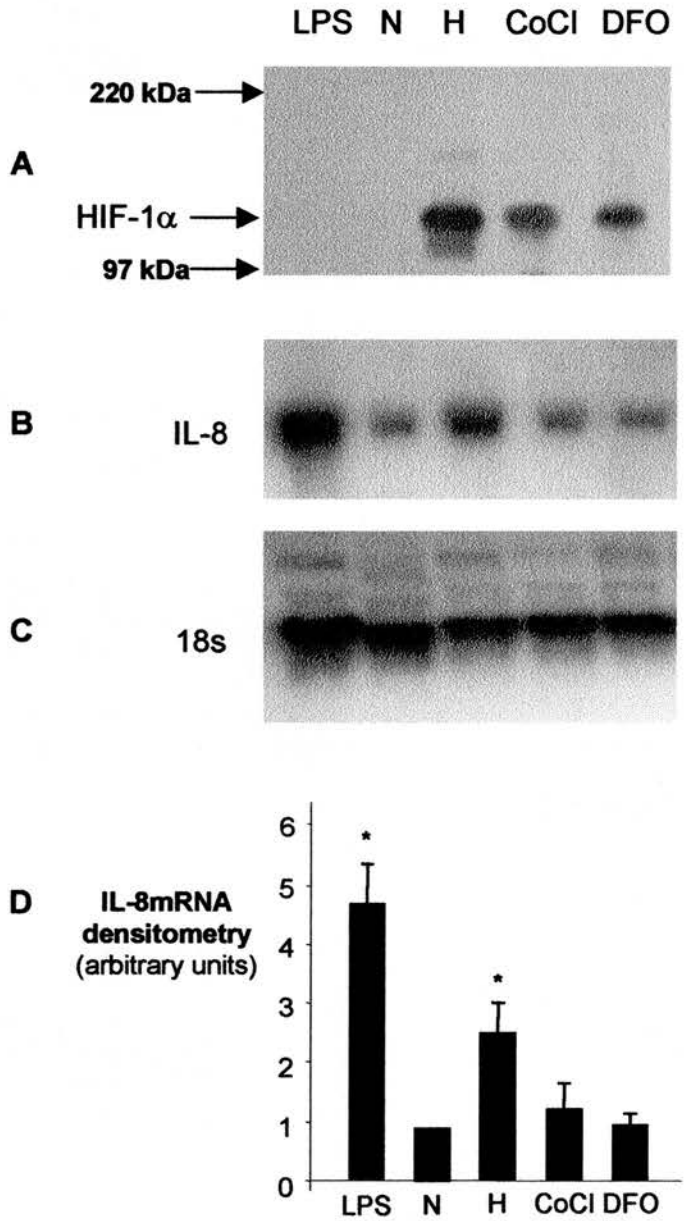
6.2.1. Cobalt and Desferrioxamine do not increase IL-8mRNA expression

Cobaltous ions and iron chelators, through mechanisms which remain unclear, are able to mimic hypoxic induction of HIF-1α protein expression and activation of a range of HIF-1-mediated target genes (Goldberg, 1988; Wang and Semenza, 1993).

Macrophages were exposed to normoxia, hypoxia, CoCl (100μM), desferrioxamine (DFO) (1 mM) or LPS (1 μg/ml) for 1 hour (**Figure 6.2.1**). Immunoblotting for HIF-1α revealed expression in hypoxic cells and those treated with CoCl and DFO, with no expression in normoxic or LPS-treated cells. In contrast to hypoxia and LPS however, neither CoCl nor DFO increased IL-8 mRNA expression.

These data suggest that HIF-1 may not be implicated in the hypoxic induction of IL-8. To further explore a possible functional role for HIF-1 in IL-8 gene transcription, experiments were planned in which a reporter-linked IL-8 promoter, would be transfected into human monocyte-derived macrophages. By transfecting IL-8 DNA with a mutated HIF-1 site, the functional nature of this putative site may be established. Indeed, this methodology would allow studies of the functional roles of the NF-κB, AP-1 and C/EBP–β sites in hypoxic IL-8 induction.

Figure 6.2.1

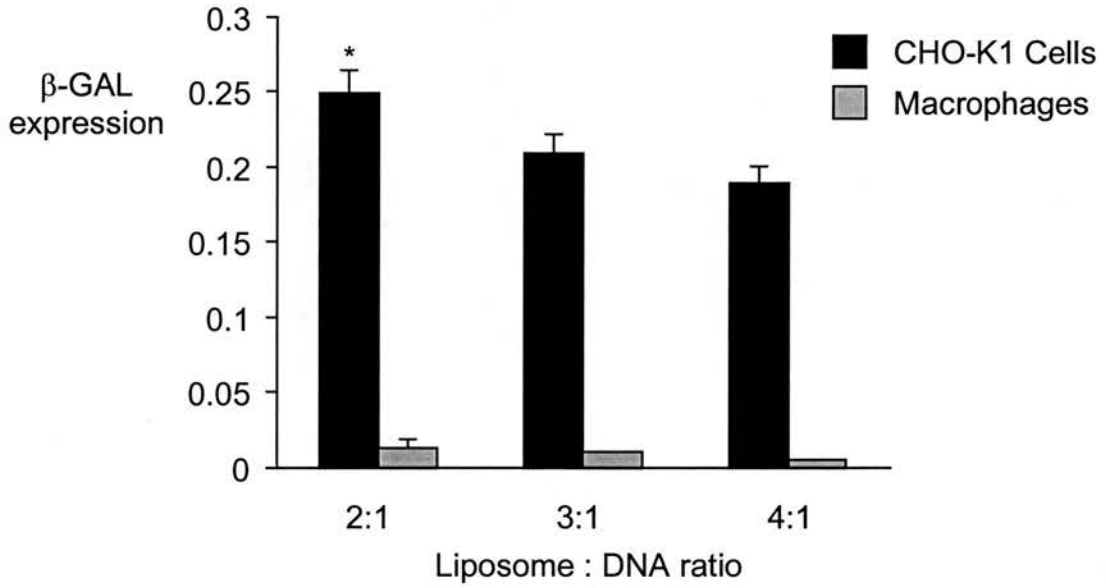


Legend for Figure 6.2.1. Hypoxia induces HIF-1a protein expression in human macrophages. Macrophages were cultured under normoxic (N) or hypoxic (H) conditions or with LPS (1 μg/ml) CoCl (100 μM) or DFO (1 mM) for 2 hrs. Hypoxia, CoCl and DFO induced HIF-1α expression (**A**). IL-8 mRNA expression from treated macrophages was assayed by northern blotting, with subsequent verification of equal RNA loading determined by re-probing with an 18s house-keeping probe (**B** and **C**).
Densitometry, corrected for 18s expression, showed that LPS and hypoxia, but not CoCl and DFO, significantly upregulate IL-8 mRNA expression (**D**).
Immunoblotting of whole protein lysates with a HIF-1α antibody was performed on n=3 separate experiments. The blot shown was performed by Dr Michael Weisener, Wellcome Trust Centre for Human Genetics, Oxford. The northern analysis was performed on n=3 separate experiments. Each value in Figure 6.1.2D represents mean ± SEM. *P<0.02 compared to normoxia.

6.2.2. Cationic liposome-mediated transfection of human monocyte-derived macrophages is inefficient.

Human monocyte derived macrophages were transfected with a pSV-β-Galactosidase vector using the cationic liposome Tfx-10 (Promega) as described in **Chapter 2**. Three different liposome : DNA ratios were used; 2:1, 3:1 and 4:1. Post-transfection, cells were cultured for 18 hours under normoxic conditions. CHO-K1 cells, known to be readily transfectable, were used as a positive control. Cell viability, as measured by Trypan blue exclusion, was >95% in both cell types when transfected at a charge ratio of 2:1 or 3:1. In macrophages, viability was reduced to 80%, and in C4.5 cells 85%, at a charge ratio of 4:1. The expression of β-GAL protein in the cell lysate was measured by ELISA, and adjusted for total protein. At all three charge ratios, macrophages expressed markedly less β-GAL compared to CHO-K1 cells (**Figure 6.2.2**). At a charge ratio of 2:1, β-GAL expression in CHO-K1 cells was optimal (0.25μg/μl), and was 20-fold greater than the corresponding value in macrophages.

Figure 6.2.2



Legend for 6.2.2. Liposome mediated transfection in CHO-K1 cells and macrophages. Cells were transfected at three different charge ratios. Monocyte-derived macrophages expressed markedly less b-GAL compared to CHO-K1. A liposome ratio of 2:1 was significantly more efficient than 3:1 or 2:1. This experiment was performed once in triplicate wells. Data is presented as mean ± SD. *P<0.05 compared to all other transfections.

Hence, liposome-mediated transfer of DNA to human monocyte-derived macrophages was comparatively inefficient, and was thought not to be a useful method for investigating IL-8 gene regulation in these cells.

An alternative approach was employed, using engineered HIF-1α - deficient (Ka13) and HIF-1α - expressing (C4.5) CHO-K1 cell-lines (a kind gift from Prof. Peter Ratcliffe, Oxford). The derivation and characterisation of this cell line is well described (Wood et al., 1998). Briefly CHO-K1 cells were stably transfected (grown on selective media) with plasmids bearing HREs linked to genes encoding immunoselectable cell surface markers (CD2 and E-Selectin). Cells were mutagenised by exposure to 3-chloro-7-methoxy-9-(3-[chloroethyl]-amino propylamino)-acridine dihydrochloride. Derived cells which displayed reduced or absent hypoxia-inducible marker expression were further screened and one clone (Ka13) revealed absent HIF-1α expression. A clone which strongly expressed hypoxia-inducible surface markers (C4.5) was also selected as a control cell population. Hypoxic Ka13 cells fail to upregulate several endogenous genes in which HIF-1α has been implicated (Wood et al., 1998)

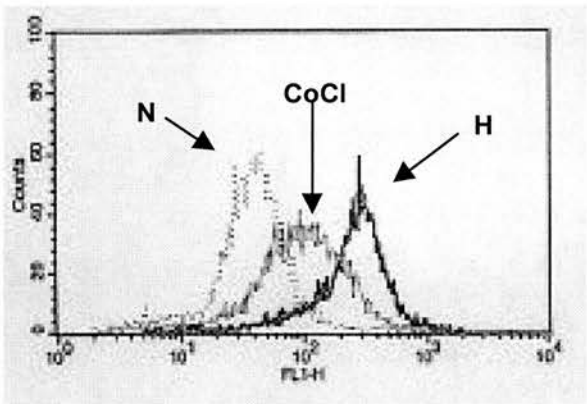
6.2.3 C4.5 cells upregulate CD2 and E-selectin following hypoxia and CoCl treatment

In order to verify the C4.5 cell phenotype and ensure that the reported effects of hypoxia in this cell could be reproduced in the conditions used in my experiments, cells were incubated for 18 hours under normoxic or hypoxic conditions, or with CoCl (100 μM). Cells were immunostained with mouse anti-human monoclonal antibodies to CD2 and E-selectin and analysed by FACS. Antibodies to human CD5 (a T cell marker) and MOPC (a mouse myeloma IgG1 marker) were employed as negative controls. Both hypoxia and CoCl increased CD2 and E-selectin expression in C4.5 cells, compared to normoxic controls (**Table 6.2.3 and Figure 6.2.3**).

Table 6.2.3

	Mean log fluorescence with antibody				
	FB	CD5	MOPC	CD2	E-selectin
N	4.0	4.0	3.6	47	10.5
	4.2	4.1	3.6	50	13
H	4.8	4.6	4.2	277	107
	5	4.3	4.4	289	97
CoCl	3.5	3.7	121	48	3.4
	3.7	3.7	128	54	3.1

Figure 6.2.3



Legend for Table 6.2.3 and Figure 6.2.3. Verification of the C4.5 cell phenotype. Both hypoxia (H) and CoCl upregulate surface expression of CD2 and E-selectin compared to normoxia (N). (Table 6.2.3). Flow buffer, CD5 and MOPC were negative controls. A graphical representation of the data for CD2 is shown in Figure 6.2.3, a flow-cytometry histogram of cell count versus log fluorescence. This experiment was performed once, in duplicate. Both values are shown in Table 6.2.3.

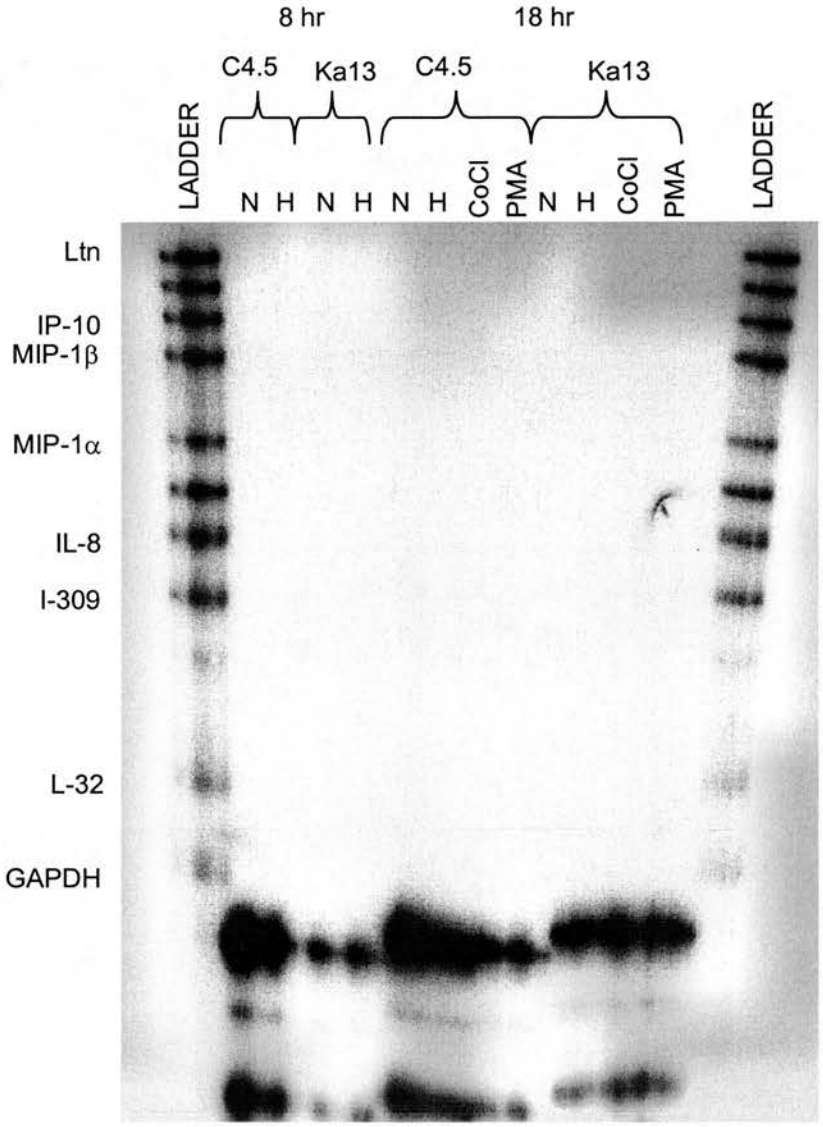
6.2.4. Detection of IL-8 and other chemokines in C4.5 and Ka13 cells

It is not known if CHO-KI cells express IL-8 or related homologue. There are no entries in the GenBank database pertaining to Chinese hamster IL-8. Hence, total RNA from normoxic, hypoxic and PMA-treated C4.5 cells was 'screened' for IL-8 mRNA by northern blotting using the human IL-8 cDNA probe, by RT-PCR using both human and rabbit IL-8 PCR primers. None of these techniques yielded products consistent with IL-8 transcript (data not shown). RNase-protection assay (RPA) using human riboprobes to selected chemokines (as described in Chapters 2 and 5) was also attempted. Wood et al., 1999 have used this technique to study expression of several genes using Chinese Hamster, murine or human RNA probes in C4.5 and Ka13 cells. However, no bands other than the housekeeping GAPDH and L32 were observed by RPA as shown in **Figure 6.2.4.**

These data suggested either that CHO cells do not express IL-8, or that Chinese hamster IL-8 transcript does not bear sufficient cross-species homology to allow detection by the

human or rabbit IL-8 reagents used. C4.5 and Ka13 cells were therefore transfected with the human IL-8 promoter.

Figure 6.2.4.



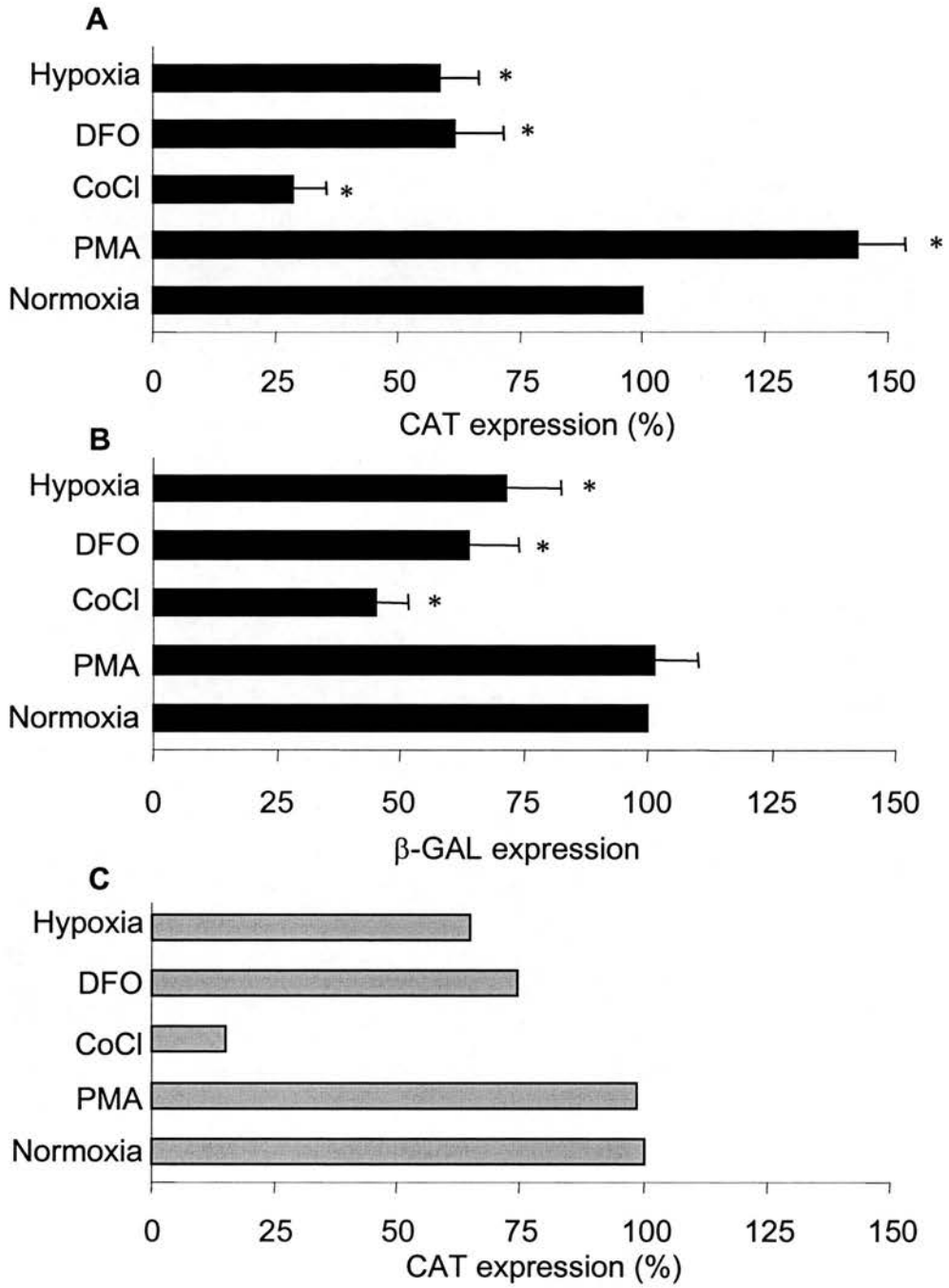
Legend for Figure 6.2.4. . RNase protection assay using multiple human RNA probes in C4.5 and Ka13 CHO-K1 cells. Human riboprobes failed to detect chemokine mRNA species from CHO cells. Bands were not visualised even following prolonged radiographic exposure. This assay was performed on RNA samples obtained from a single experiment.

6.2.5 and 6.2.6. IL-8-Reporter-linked expression in C4.5 and Ka13 cells

Cells were co-transfected with the CAT-linked IL-8 promoter and a β-Gal control vector (vector maps shown in **Figure 2.2.12.1**) and subsequently incubated under normoxic or hypoxic conditions, or with DFO, CoCl or PMA, for 18 hours. Cell lysates were assayed for CAT (and β-Gal) protein. **Figure 6.2.5** shows that compared to normoxia (expressed as 100%), hypoxia, DFO and CoCl all significantly inhibited CAT protein expression (58±8%, 61±10% and 26±6% respectively; $P<0.02$). PMA significantly increased CAT expression (138±10 %; $P<0.02$). However, an unexpected finding was that hypoxia, DFO and CoCl also significantly inhibited β-Gal control vector expression (61±10 %, 58±10 % and 44±5 % respectively; $P<0.02$). PMA had no significant effect on β-Gal expression (101±7 %). As an additional control experiment, cells were transfected with pCAT-Control Vector. This vector contains SV40 promoter and enhancer sequences (vector maps are shown in **Figure 2.2.13**), and is designed to induce strong constitutive expression in eukaryotic cells. Again, hypoxia, DFO and CoCl all inhibited CAT expression to a similar degree to IL-8 transfected cells (**Figure 6.2.5**).

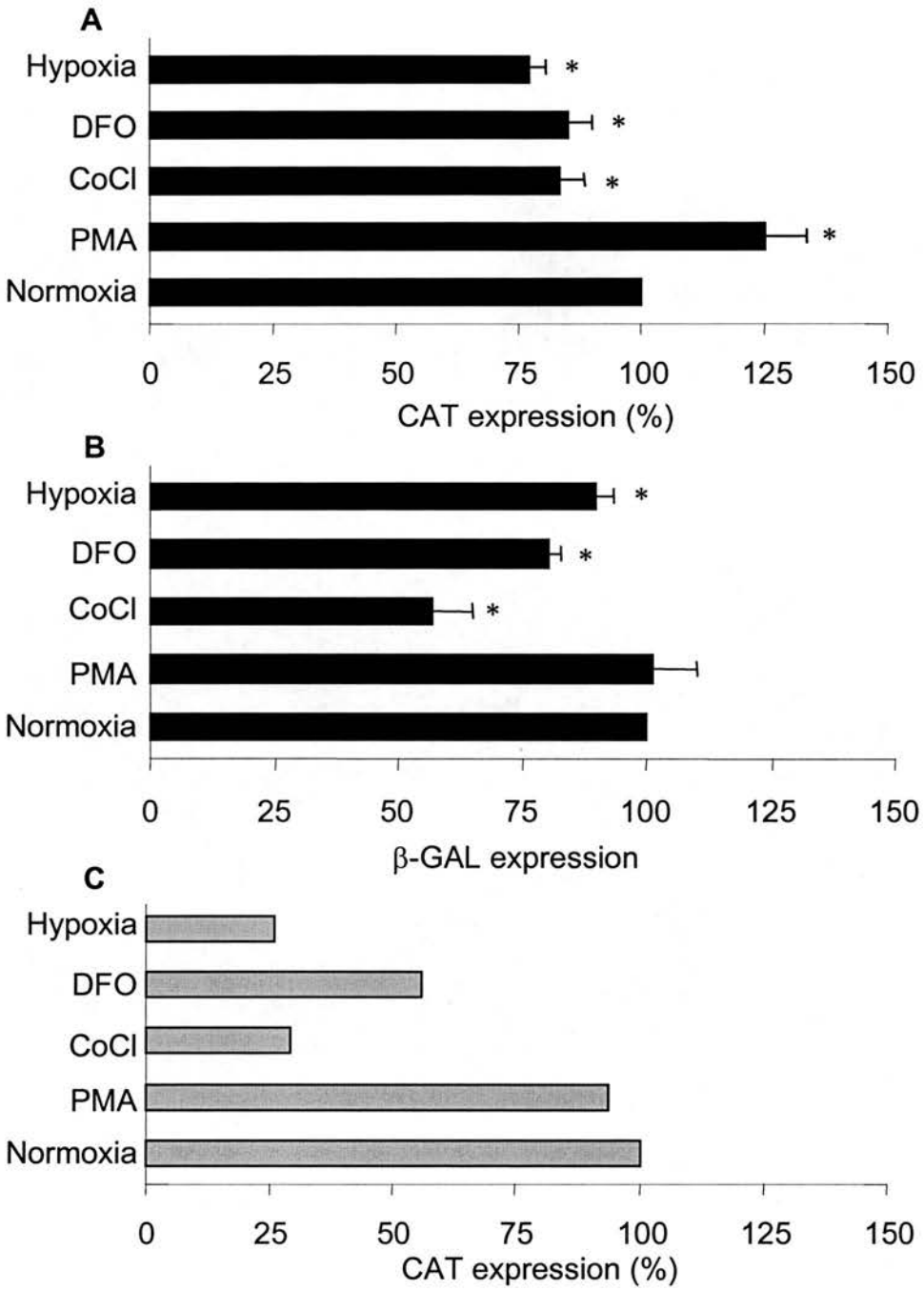
The pattern of reporter protein expression from transfected Ka13 cells was similar to that observed with C4.5 cells; hypoxia, DFO and CoCl all significantly inhibited CAT and β-Gal expression in cells co-transfected with CAT-linked IL-8 promoter and β-Gal control vector (**Figure 6.2.6**). Similarly, Ka13 cells transfected with pCAT-control vector expressed significantly less CAT protein following hypoxia DFO and CoCl treatment than to normoxia.

Figure 6.2.5



Legend for Figure 6.2.5. IL-8 reporter-linked expresion in C4.5 cells. C4.5 (HIF-1 expressing) cells were co-transfected with the CAT-linked IL-8 promoter (**A**) and a pSV-β-Galactosidase vector (**B**). Cells were cultured in normoxic or hypoxic conditions, or with PMA (100 nM), CoCl (100 μM) or DFO (1mM) for 18 hrs. Results are expressed as mean SEM. * $P<0.02$, n=3 experiments performed in duplicate. As a further control (see text), C4.5 cells were transfected with a pCAT control vector and treated as above for 18 hrs (**C**). Results represent mean of n=2 experiments performed in duplicate.

Figure 6.2.6



Legend for Figure 6.2.6. IL-8 reporter-linked expresion in Ka13 cells. As with C4.5 cells Ka13 (HIF-1 deficient) cells were co-transfected with the CAT-linked IL-8 promoter (**A**) and a pSV-β-Galactosidase vector (**B**). Cells were cultured in normoxic or hypoxic conditions, or with PMA (100 nM), CoCl (100 μM) or DFO (1mM) for 18 hrs. Results are expressed as mean SEM. * $P<0.02$, n=3 experiments performed in duplicate. As an additional control, Ka 13 cells were separately transfected with a pCAT control vector and treated as above for 18 hrs (**C**). Results represent mean of n=2 experiments, performed in duplicate

These experiments clearly show that the inhibitory effects of hypoxia, DFO and CoCl on reporter protein expression in CAT-linked IL-8 promoter transfected cells cannot be attributed to an effect on the IL-8 promoter. There are several possibilities that may explain apparent global reduction in expression of reporter protein as observed in these studies:

i) Experimental treatment with hypoxia, CoCl and DFO significantly reduced cell viability

ii) Hypoxia, CoCl and DFO interfere with CAT (and β-GAL) protein translation, stability or detection by ELISA.

iii) Hypoxia, CoCl and DFO interfere with basal transcription machinery. In this case, not only eukaryotic but also prokaryotic transcriptional apparatus must be repressed by the experimental treatment.

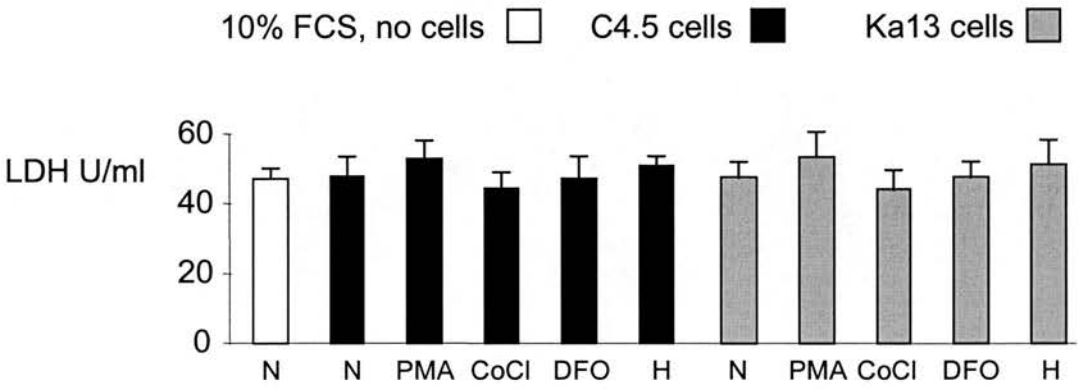
6.2.7 Inhibition of reporter protein expression is not due to reduced cell viability

Cell viability of transfected cells following 18 hours hypoxia, DFO or CoCl treatment was assessed by trypan blue exclusion, which consistently revealed >95% viability. However to further explore the possibility that hypoxia, CoCl and DFO were reducing reporter protein expression as a result of cell death, LDH levels were was assayed in the supernatant following 18 hours culture. There was no increase in supernatant LDH levels under experimental conditions **(Figure 6.2.7)**.

6.2.8. Hypoxia increases intracellular LDH expression in C4.5, but not Ka13 cells

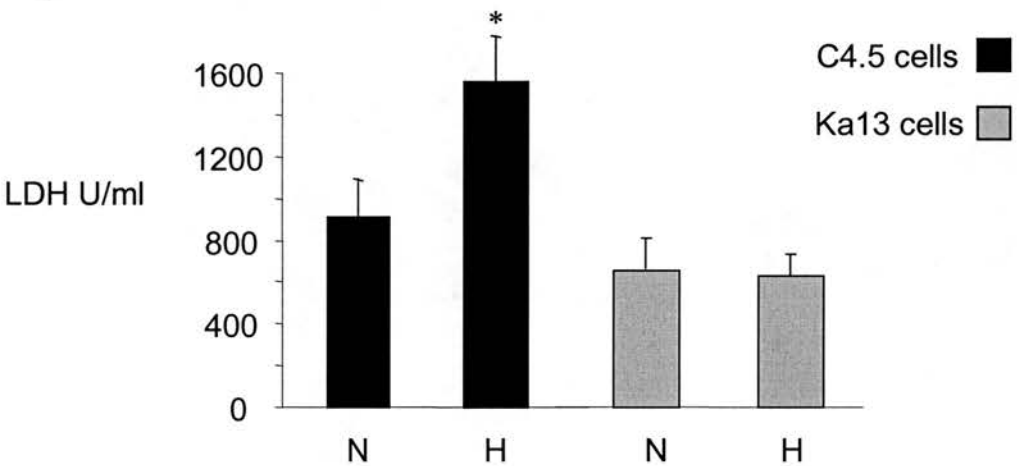
LDH is a glycolytic enzyme known to be upregulated by hypoxia. Previous pharmacological studies have shown that HIF-1 is likely to be involved in hypoxic-induction of LDH (Firth et al., 1994). Intracellular LDH levels were assayed in lysates from C4.5 and Ka13 cells cultured in normoxic or hypoxic conditions. This showed that in C4.5 cells, hypoxia significantly increased intracellular LDH protein levels compared to normoxic controls (1585±202 v 908±191 U/ml; $P<0.02$). In Ka13 cells LDH levels in hypoxic and normoxic cells were identical (605±197 v 594±54 U/ml), evidence that hypoxic upregulation of LDH is HIF-1α - dependent.

Figure 6.2.7



Legend for Figure 6.2.7. Viablity of CHO cells assessed by LDH assay in cell supernatants. Supernatants from transfected C4.5 and Ka13 cells incubated in normoxia (N), hypoxia (H) or with PMA (100 nM), CoCl (100 μM) or DFO (100 mM) for 18 hours in 10% FCS were assayed for LDH content. Medium with 10% FCS was also assayed for background LDH levels. No significant increase over background levels was detected. Data is presented as mean ± SD. n=1, performed in triplicate wells

Figure 6.2.8

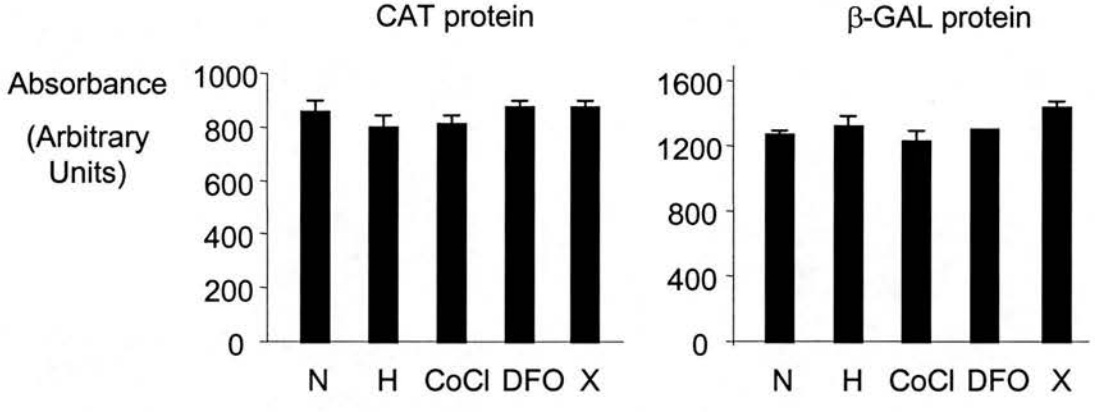


Legend for Figure 6.2.8. Upregulation of intracellular LDH by hypoxia in C4.5 and Ka13 cells. C4.5 and Ka13 cells were incubated in normoxic (N) or hypoxia (H) for 18 hours and lysed in 0.1% Triton-X100. Intracellular LDH was significantly increased in C4.5 cells but not in Ka13 cells. Data is presented as mean ± SEM. n=3 experiments, performed in triplicate wells. $P<0.002$.

6.2.9. Inhibition of reporter protein expression is not due to reduced protein stability or impaired detection.

The reporter assays for both CAT and β-Gal depend upon measurement of reporter protein levels by ELISA. Recombinant CAT and β-Gal protein from E.Coli (Boehringer Mannheim) was reconstituted at 0.5 ng/ml in 1ml of medium with 10% FCS. The medium was then incubated under normoxic or hypoxic conditions, or with 100 μM CoCl or 1 mM DFO for 18 hours. Measured levels of both recombinant CAT and β-Gal protein following hypoxic exposure or spiking with CoCl or DFO were similar to normoxic controls (**Figure 6.2.9**). This suggests that neither protein stability nor detection by ELISA were influenced by the experimental treatments.

Figure 6.2.9



Legend for Figure 6.2.9. Effect of Hypoxia, CoCl and recombinant CAT and β-Gal protein detection by ELISA. Recombinant CAT and β-Gal protein (0.5 ng/ml) was incubated under normoxic or hypoxic conditions, or with 100 μM CoCl or 1 mM DFO, for 18 hours. Standard ELISA was performed, and absorbance measured at 405 nm. For comparison, absorbance from an aliquot of freshly reconstituted protein (0.5 ng/ml) is also shown (X). Incubation of protein with the various treatments did not affect detection by ELISA. Data is presented as mean ± SD. n=1 experiment performed in triplicate

In the absence of evidence for reduced cell viability, reduced protein stability or impaired detection, it is possible that hypoxia, DFO and CoCl influence basal eukaryotic and prokaryotic transcriptional machinery. This is discussed further in the Discussion.

6.3 DISCUSSION

In these experiments the potential role of HIF-1 in the hypoxic regulation of IL-8 in macrophages was explored. Human macrophages were shown to express HIF-1α under hypoxic conditions, and following treatment with coboltous ions and DFO. However neither CoCl nor DFO significantly upregulated IL-8 mRNA expression. In HIF-1 - deficient CHO cells transfected with a CAT-linked IL-8 promoter, hypoxia, CoCl and DFO all significantly inhibited CAT expression compared to normoxia. However, identical results were seen with the control β-Gal vector and with a pCAT vector harbouring SV40 promotor and enhancer sequences. The inhibited protein expression was not a result of reduced cell viabilty as determined by trypan blue exclusion and LDH assay of cell supernatants.

Pharmacological manipulation of HIF-1 expression has provided a useful tool for studying the potential role of HIF-1 in gene regulation. Coboltous ions and iron chelators such as DFO mimic hypoxia in both induction of HIF-1α protein and activation of HIF-1 mediated target genes such as erythropoietin (Goldberg et al., 1988; Wang and Semenza, 1993), VEGF (Goldberg et al., 1994) transferrin receptor (Lok et al., 1999) LDH-A (Firth et al., 1994), PDGF-β (Gleadle et al., 1995) endothelin-1 (Hu et al., 1998) and NO synthase (Melillo et al., 1997).

The mechanisms by which cobaltous (and other divalent transition metal) ions and the iron chelator DFO induce HIF-1 are unclear (**Chapter 1.5.5**). To date however, there are no reported examples of hypoxia inducible genes in which HIF-1 is clearly implicated (as determined by transfection of mutated or deleted reporter-linked promoter sequences), but in which neither divalent cations nor iron chelators mimic the hypoxic effect. Conversely, both CoCl and DFO may influence gene regulation via pathways independent of HIF-1 (Wenger, 2000). In CHO cells, CoCl upregulates haemoxygenase-1 (HO-1) in a HIF-1 independent manner, in association with increased DNA binding of Nrf2, a recently characterised member of the AP-1 family of bZIP transcription factors (Gong et al., 2001). The finding that neither CoCl nor DFO increased IL-8 mRNA, at a dose widely used in studies of hypoxic signalling, suggests that HIF-1 is unlikely to play a role in IL-8 synthesis in human macrophages.

Recently inflammatory stimuli such as TNF-α, and IL-1β have been shown to induce HIF-1α expression (Haddad and Land, 2001) and increases DNA-binding activity (Hellwig-Burgel et al., 1999) in epithelial and endothelial cell lines. In the macrophages studied herein, HIF-1α expression was not induced by LPS. Whether other inflammatory stimuli such as cytokines induce HIF-1 expression in macrophages is not known. However, it could be hypothesised that for macrophages, which must adapt to miroenvironmental changes during migration to the inflammatory site, it may be advantageous to differentiate hypoxic from infective stimuli.

To further explore a role for HIF-1 in IL-8 transcription, experiments were performed in a HIF-1α deficient cell line. The C4.5 (HIF-1α expressing) cell line has been stably transfected with a plasmid bearing multiple HREs linked to genes encoding CD2 and E-selectin. A mutagenised clone of this cell line, the Ka13 cell line, does not express HIF-1α and hence does not express CD2 or E-selectin following hypoxia (Wood et al., 1998). These cells have proved to be a useful tool for studying the role of HIF-1 in the hypoxic regulation of genes including HO-1, Glucose transporter-1 (Glut-1) and VEGF (Wood et al., 1998). CHO cells did not appear to express IL-8 mRNA as detectable by human and rabbit PCR primers, cDNA probes and riboprobes The cells were therefore transfected with a CAT-linked IL-8 promoter.

The results demonstrate that in C4.5 transfected with a CAT-linked IL-8 promoter, hypoxia, DFO and CoCl all significantly inhibited CAT expression compared to normoxic controls. This phenomenon however cannot be attributed to a specific effect on the IL-8 promoter. Expression of the co-transfected β-Gal vector, which is under the control of the constitutively driven SV40 early promoter and enhancer, was similarly inhibited by hypoxia, CoCl and DFO. Similarly, transfection of a pCAT control vector under the control of the SV40 early promoter and enhancer resulted in reduced CAT expression following hypoxia, CoCl and DFO. Furthermore, this effect cannot be attributed to HIF-1-mediated hypoxic signal, since Ka13 (non HIF-1 - expressing) cells responded identically. Treatment with PMA, a C-kinase activator and known IL-8 activator (Schondorf et al., 1993; Okamoto et al., 1994; Smyth et al., 1991) did induce increased CAT expression in IL-8 promoter transfected cells, with no effect on β-GAL or

pCAT control vector transfected cells. This demonstrated that CHO cells possess the capacity to induce the IL-8 promoter if appropriately stimulated. Furthermore, the observation that intracellular LDH was upregulated in a HIF-1 dependent manner, as has previously been described (Semenza et al., 1994; Firth et al., 1995), suggests an appropriate cellular response to hypoxia.

A number of possible explanations for these unexpected findings were explored. Reporter assays measure expression at the protein level. Hence in addition to factors that influence gene promoter or enhancer elements, cell viability, reporter protein stability and detection, protein translation, reporter mRNA stability and the general transcriptional machinery may all influence the assay result. Experiments were performed to address some of these possibilities. The inhibitory effects of hypoxia, CoCl and DFO would be accounted for if these treatments reduced cell viability. This however was not the case, since cell viability as measured by both trypan blue exclusion and LDH activity in cell supernatants was unaffected. Similarly experiments in which recombinant CAT and β-GAL protein was co-incubated with CoCl and DFO prior to ELISA suggested that the effect was not due to reduced CAT or β-GAL protein stability or impaired protein detection.

The inhibitory effects of hypoxia, CoCl and DFO may therefore occur at the mRNA level, either through impaired reporter mRNA translation to protein, impaired mRNA stability, or through inhibition of general transcriptional machinery. Experiments that address these specific issues have not been performed. The transcription of transfected reporter-linked genes, as with transcription of all native genes in eukaryocytic cells, is dependent upon the activation of basal transcriptional apparatus. This process consists of the binding of general transcription factors (starting with TFIID) to the TATA sequence. This results in recruitment of RNA polymerase II to the DNA promoter, an essential event in gene transcription. Inhibition of general transcriptional machinery would therefore be a possible mechanism for the inhibition of reporter expression.

Prokaryotic promoters are commonly used in as 'control' in transfection experiments, on the basis that prokaryotic transcriptional machinery should function constitutively and

independently of eukaryotic regulation. Previous studies have demonstrated that prokaryotic vector DNA can alter eukaryotic transgene expression *in vivo*. In a transgenic alpha-fetoprotein mouse model, plasmid prokaryotic DNA elements were found to be responsible for inhibiting expression of the eukaryotic transgene *in vivo* (Hammer et al., 1987). Whilst these *in vivo* findings support the notion that prokaryotic and eukaryotic transcriptional control is not mutually exclusive, the fact remains that viral promoters are used extensively *in vitro*, including hypoxia experiments, without reports of eukaryotic interference (Semenza et al., 2000B and references therein).

The effects of hypoxia, CoCl and DFO on reporter expression in transfected cells are not easily explained and require further study. For example, it would be of interest to investigate the effect of hypoxia on PMA-stimulated cells, to observe if the inhibitory effects on reporter expression will over-ride a positive stimulus. Furthermore, CHO cells transfected with alternative viral-controlled expression vectors, such as cytomegalovirus (CMV) or adenoviral vectors would provide information on the specificity of the observed effects to the SV-40 construct.

SUMMARY

The original catalyst for much of the work presented herein has come from clinical studies of patients at-risk of developing ARDS, in which it has been shown that raised intrapulmonary levels of IL-8 following multiple trauma are associated with progression to this catastrophic form of lung injury. The mechanisms by which intrapulmonary IL-8 may be raised in this context are unknown but the observation that the high levels of IL-8 were detected within a few hours (range 0.75 – 4 hr) of trauma suggest that events occurring in the immediate aftermath of the acute insult play a role in stimulating IL-8 generation. I hypothesised that clinically relevant potential stimuli in this scenario included acute stress mediators such as catecholamines and neuropeptides and acute physiological events, principally tissue hypoxia and resuscitation-associated hyperoxia.

In *in vitro* studies of human monocyte-derived macrophages, adrenalin, substance P and MIF had no effect on IL-8 protein release at 2 hours, an early time point chosen to reflect the timing of clinical samples taken from trauma patients. Acute hypoxia (PO_2 ~ 3.5 KPa) increased IL-8 protein release by 1.8-fold by 2 hours and steady-state IL-8 mRNA expression by 30 min compared to normoxic controls. The introduction of hyperoxia, as a second stimulus or 'hit' following hypoxia was a more potent stimulus for IL-8 protein generation than hypoxia or hyperoxia alone.

The effects of hypoxia / hyperoxia on IL-8 generation were further studied in a rabbit model of acute lung injury. Localised bronchoscopic instillation of HCl into the left lower lobe of an anaesthetised ventilated rabbit resulted in significantly increased i) IL-8 mRNA and protein expression, ii) neutrophil infiltration into alveolar airspaces and iii) lung leak as measured by Evans blue labelled-albumin in the directly injured lung compared to the contralateral 'indirectly' injured lung. Systemic hypoxaemia was induced by reducing in the inspiratory oxygen fraction. Compared to normoxic controls (arterial PaO_2 ~ 11 KPa), acute hypoxia (PaO_2 ~ 5 KPa) for up to 2 hours increased intrapulmonary IL-8 mRNA but not protein expression in the acid-injured lung. Delivery of 100% oxygen for 2 hours (PaO_2 ~ 60 KPa) following acute hypoxia (a multiple-hit), increased both intrapulmonary IL-8

mRNA and IL-8 protein levels. The increase in IL-8 protein was attenuated if the reoxygenation phase was controlled to return arterial PO_2 to normoxic levels (~ 11 KPa). These data support the hypothesis that acute hypoxia / hyperoxia are clinically relevant stimuli that can generate raised levels of intrapulmonary IL-8.

The mechanisms by which hypoxia may rapidly increase IL-8 mRNA expression in monocyte-derived macrophages was further studied *in vitro*. The rapidity of the response (30 mins) suggested an increase in gene transcription. Electromobility gel-shift assay revealed that hypoxia increased nuclear levels of the IL-8 promoter-binding transcription factors AP-1 and CEBP-β, but not NF-κB, by 15 min exposure. Hypoxia induced macrophage expression of HIF-1α, a critical regulator of hypoxic adaptive responses. However cobalt chloride and desferrioxamine (DFO), HIF-1α-inducing hypoxia mimics, did not upregulate IL-8 suggesting that IL-8 transcription may be HIF-1 independent. Finally it was demonstrated that in contrast to IL-8, hypoxia inhibited expression of a panel of chemokines and cytokines including MCP-1, MIP-1α, MIP-1β and TNF-α. Both the pattern of chemokine expression and transcription factor activation observed with hypoxia differed from that induced by bacterial lipopolysaccharide (LPS), which potently activated NF-κB and upregulated several chemokine genes. This implies potentially distinct adaptive responses to hypoxia and infection in the macrophage.

Future studies

Several observations made in the course of this thesis are worthy of further study. The majority of the *in vitro* studies were performed in monocyte-derived macrophages, serving as model of the resident alveolar macrophage. There may however be significant differences in functional response between the cell phenotypes and complementary studies in alveolar macrophages from healthy human donors would address this issue. The observation that hypoxia rapidly increased steady-state IL-8 mRNA expression in association with increased nuclear expression of regulatory transcription factors strongly suggests an increase in gene transcription. However this requires confirmation by nuclear run-off assay. The effect of hypoxia on IL-8 mRNA stability may also be tested in studies with the transcriptional inhibitor actinomycin-D.

The observations from EMSA studies implicated AP-1 and C/EBP-β, but not NF-κB or HIF-1 in the hypoxic regulation of IL-8. Reporter-linked transfection studies were hampered through difficulty transfecting primary macrophages using standard liposome-mediated transfer. A different approach may be to employ adenovirus infection to deliver the IL-8 promoter to macrophages. Alternatively, although THP-1 cells appeared not to respond to hypoxia, other human monocyte/macrophage cell lines such as U937 or Mono-Mac-6 could be 'screened' for hypoxic-induction of IL-8. Such cell lines are generally more amenable to transfection through standard techniques. However, the differential regulation of a number of chemokines and cytokines, several of which have in common transcription factor binding sites for AP-1, C/EBP-β and NF-κB, suggests the involvement of other regulatory factors. The most obvious candidate would be HIF-1. However the finding that neither cobalt chloride nor desferrioxamine induced IL-8 transcription is evidence against a role for HIF-1 in IL-8 gene regulation. This raises the possibility of a regulatory mechanism that overrides, or more likely complements transcription-factor control in selective gene transcription in response to hypoxia. Histone modification represents one potential mechanism and in particular there is evidence that histone acetylation may differentially regulate chemokines (Fusunyan et al., 1999; Wen and Wu, 2001). The effect of hypoxia on histone acetylation status in macrophages would represent an exciting area for future study.

Whilst the observations reported demonstrate that hypoxia and hyperoxia generate IL-8 synthesis in macrophages and in lung tissue *in vivo*, the direct relevance of these findings to the clinical setting of multiple trauma is debatable. Firstly, although hypoventilation, atelectasis, and contusion are all likely to feature acutely in trauma victims, the presence of associated widespread alveolar hypoxia is not certain. Alveolar hyperoxia in contrast would be very likely to occur in the resuscitation phase following trauma and is therefore of direct clinical relevance. Direct measurement of tissue oxygenation in the acute clinical setting is feasible but presents significant practical obstacles. Animal models of trauma present logistical and ethical constraints, hence our use of a direct acid-injury model. However, some aspects of the trauma clinical scenario, such as haemorrhage, hypoventilation,

atelectasis and hyperoxic resuscitation may be reproduced in an animal model. This would present the opportunity to study the effects of tissue hypoxia and hyperoxia not only in the development of ARDS but also the inflammatory response associated with MODS.

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APPENDICES

Appendix 1A. American College of Chest Physicians and Society of Critical Care Medicine (ACCP/SCCM) definition for SIRS. Bone et al., 1992.

Systemic Inflammatory Response Syndrome (SIRS)	
A severe clinical illness with the presence of two or more of the following criteria :	
Temperature	>38°C *or* <36°C
Tachycardia	>90 beats / min
Tachypnoea	>20 breaths / min *or* $PaCO_2$ < 4.25 KPa
Altered white blood cell count	>12 x 10^9/L *or* <4 x 10^9/L *or* >10% band forms on peripheral blood film

Appendix 1B. The North American / European Concensus Criteria for ALI and ARDS. Bernard et al., 1994

	Acute lung injury (ALI)	**ARDS**
Timing	Acute onset	
Oxygenation	PaO_2 / FiO_2 < 40 KPa regardless of PEEP	PaO_2 / FiO_2 < 26.6 KPa Regardless of PEEP
Radiography	Bilateral infiltrates on chest X-ray	
Pulmonary artery wedge pressure	< 18 mmHg with no evidence of left atrial hypertension	

Appendix 1C

Mediators assayed in blood and airspaces of patients at-risk of ARDS (heterogenous risk factors)

Biological Mediator		References
Cytokines	TNF-α	Marks et al., 1990; Parsons et al., 1992; Raponi et al., 1992; Hyers et al., 1991, Roten et al., 1991
	IL-1β	Siler et al., 1989; Pugin, 1996
	IL-8	Jorens et al., 1992; Donnelly et al., 1993; Torre et al., 1993; Hirani et al., 2001
	IL-1ra	Parsons et al., 1997
	IL-10	Parsons et al., 1997
Bacterial products	Endotoxin	Parsons et al., 1989; Donnelly et al., 1994A; Goldie et al., 1995;
Lipids and metabolites of arachadonic acid	LTB_4	Stephenson et al., 1988; Davis et al., 1990; Antonelli et al., 1994; Amat et al., 2000
	Lipofuscin	Roumen et al., 1994
	C18 unsaturated fatty acids	Bursten et al., 1996
Complement	C5a	Weinberg et al., 1984; Duchateau et al., 1984
	C5b-9 terminal complex	Langlois and Gowry, 1988
Markers of neutrophil / endothelial activation	Elastase	Donnelly et al., 1995;
	E / P- selectins	Donnelly et al., 1994; Sakamaki et al., 1995
	L-selectin	Stegel et al., 2001
	vWf-Ag	Rubin et al., 1990; Moss et al., 1995; Bajaj et al., 1999
	CD11b / CD18	Laurent et al., 1994; Simms et al., 1991
Growth factors and collagen products	Type 1 procollagen	Armstrong et al., 1999
	VEGF	Thicket et al., 2001
Surfactant proteins	SP-A	Pison et al., 1992; Green et al., 1999
Iron metabolism	Ferritin	Connelly et al., 1997, Sharkey et al., 1999
Markers of oxidative stress	Hydrogen peroxide	Baldwin et al., 1986
	Catalase MnSOD	Leff et al., 1993